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UNIVERSITY OF ALBERTA

Mechanistic Studies of the Porcine Plasma Amine Oxidase Reaction

by

Araba A. Coleman

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

IN

Food Chemistry

Department of Food Science

EDMONTON, ALBERTA

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entitled **Mechanistic Studies of the Porcine Plasma Amine Oxidase Reaction**

submitted by **Araba A. Coleman**

in partial fulfillment of the requirements for the

degree of **Doctor of Philosophy**

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DEDICATION

To Mum and Dad

Abstract

Copper amine oxidases catalyze the oxidative deamination of mono-, di-, and polyamines, with stoichiometric production of aldehyde, ammonia and hydrogen peroxide. The stereochemical course of oxidation by a number of these enzymes using stereospecifically labelled (1*R*)-[^{2}H], (1*S*)-[^{2}H]tyramine or dopamine, and (2*R*)-[^{2}H], (2*S*)-[^{2}H]tyramine has been investigated with ^{1}H NMR spectroscopy. Bovine, porcine and rabbit plasma and pea seedling amine oxidases were reacted with substrates in a coupled fashion, employing NADH and liver alcohol dehydrogenase, such that the aldehyde produced was converted directly to the corresponding alcohol. The alcohol products resulting from the bovine and rabbit enzyme reactions with stereospecifically labelled C-1 tyramines contained deuterium, suggesting that the reaction proceeded with nonstereospecific removal of the C-1 proton. A similar nonstereospecificity was observed for the bovine enzyme's reaction with the stereospecifically labelled C-1 dopamines. The porcine enzyme's reaction with both C-1 labelled tyramines and dopamines showed pro-*R* specificity in the abstraction of the C-1 proton. However, the pea seedling enzyme's reaction with the same substrates showed pro-*S* specificity. All but one of the enzymes (pea seedling) exhibited a solvent exchange pathway into C-2 of the products, so that the solvent wash-in process was observed to be nonstereospecific for the porcine enzyme as against the pro-*R* specificity of the bovine enzyme. The stereochemistry of the wash-out process carried out with stereospecifically labelled (2*R*)-[^{2}H] and (2*S*)-[^{2}H]tyramines with the porcine enzyme in a coupled fashion also showed a nonstereospecific protonation at C-2, a result similar to that observed for the bovine enzyme. It was also observed that the wash-in and wash-out processes for the porcine enzyme were not the microscopic reverse of one another. This result was also observed for the bovine enzyme.

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List of Abbreviations

BPAO	bovine plasma amine oxidase
Con-A	concanavalin A
Cu-AO	copper amine oxidase
DNA	deoxyribonucleic acid
EC	Enzyme Commission
FAD	flavin adenine dinucleotide
KIE	kinetic isotope effect
V_m	maximal velocity
K_m	Michaelis constant
MAO	monoamine oxidase
NADH	nicotinamide adenine dinucleotide (reduced form)
ppm	parts per million
PSAO	pea seedling amine oxidase
PAGE	polyacrylamide gel electrophoresis
PPAO	porcine plasma amine oxidase
^{1}H NMR	proton nuclear magnetic resonance
PLP	pyridoxal phosphate
PQQ	pyrroloquinoline quinone
RPAO	rabbit plasma amine oxidase
R_f	relative mobility
SSAO	semicarbazide sensitive amine oxidase
SPAO	sheep plasma amine oxidase
SDS	sodium dodecyl sulfate
TLC	thin-layer chromatography
TOPA	3,4,6-trihydroxyphenylalanine

1. General Introduction

Amine oxidases are enzymes which are of great interest since they are involved in the biological inactivation of naturally occurring amines in the animal body (1,2). These natural substrates are compounds which belong to the group of biogenic amines, e.g. dopamine and norepinephrine, and which are formed *in vivo* during various metabolic processes (2). The occurrence of these compounds is not limited only to higher forms of life, as they also occur in plants and microorganisms.

These widely distributed enzymes catalyze the oxidative deamination of many biologically important amines, with the corresponding formation of aldehyde, hydrogen peroxide and ammonia, according to Equation 1.1:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3 \qquad [1.1]$$

The first published literature on an amine oxidase, as reported by some authors (2,3,4), was tyramine oxidase from rabbit liver, by Hare in 1928 (5). This was followed by Best's discovery of histaminase from ox or horse lung (6) in 1929. Later, an amine oxidase was isolated from hog kidney which oxidized putrescine and cadaverine (3).

Until the early 1950's, there was little interest in these amine oxidases. The general impression was that the enzymes had some possible significant physiological roles, since they oxidized and inactivated amine neurotransmitters, e.g. dopamine, norepinephrine, epinephrine and serotonin, and other amines with false neurotransmitter (sympathomimetic) properties, e.g. tyramine, octopamine, phenethylamine and tryptamine. The accidental discovery of the relationship between the amine oxidases and mental disorders led to a great number of research studies on monoamine oxidase. As reported by Tabor in 1985, studies on these enzymes are ongoing, and researchers are investigating the possible involvement of amines in some mental disorders as well as their concentrations and metabolism in nervous tissues (3). Other basic biochemical studies on the nature of the enzymes have

been reported. Amine oxidase activity has been reported in prokaryotes, e.g. various bacteria, and also in eukaryotes, such as a wide variety of plant and animal tissues.

The classification of these enzymes had been unsatisfactory until recent years. The first reported classification of amine oxidases was based on substrate specificities of the enzymes. Thus, they were referred to as monoamine and diamine oxidases (2). In 1959, a different classification (1,2), based on the sensitivity of the enzymes to particular inhibitors, was put forward after the observation that most of these enzymes lacked strict substrate specificity. Two new groups were described: those resistant to carbonyl reagents, and those inhibited by carbonyl reagents such as hydrazine. A third classification, based on inhibitor specificities, then resulted in the semicarbazide-sensitive and semicarbazide-resistant amine oxidases (2).

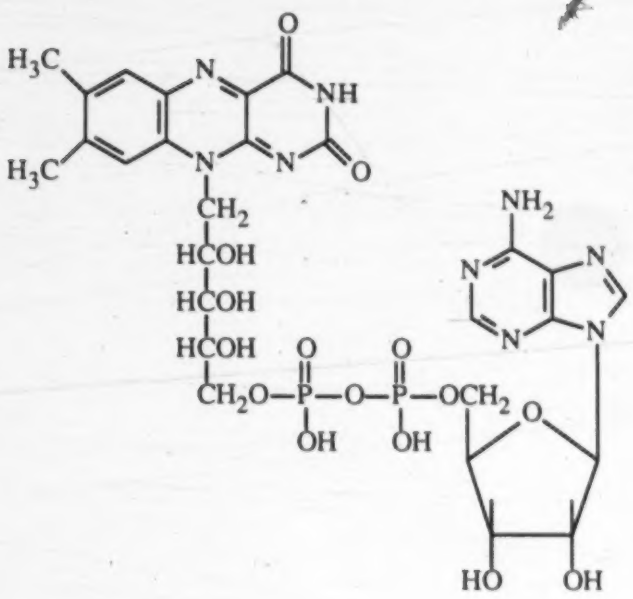
These classifications remained unsatisfactory until the nature of the prosthetic groups in the enzymes was used as a distinct parameter to distinguish these amine oxidases (8). This led to the classification of these enzymes either as flavin-containing (EC 1.4.3.4) or copper-containing amine oxidases, Cu-AO's (EC 1.4.3.6) with distinct Enzyme Commission designations.

This mode of classification has excluded those enzymes in which neither copper nor FAD has been found, but which still function as amine oxidases. These other amine oxidases, however, are extremely sensitive to the compound semicarbazide, hence the class: semicarbazide-sensitive amine oxidases (SSAO).

Flavin-containing amine oxidase

The classical monoamine oxidase (EC 1.4.3.4), containing covalently or non-covalently bound FAD (Fig. 1.1) as a prosthetic group, has been isolated from mitochondria and bacteria, respectively. Generally referred to as monoamine oxidase (MAO), the mitochondrial type is essential in the metabolism of certain neurotransmitters, such as

(a) Flavin adenine dinucleotide (FAD)



(b) Semicarbazide

(c) Diamines:

i. $NH_2(CH_2)_4NH_2$ putrescine

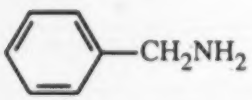
ii. $NH_2(CH_2)_5NH_2$ cadaverine

(d) Polyamines:

i. $NH_2(CH_2)_3NH(CH_2)_4NH_2$ spermidine

ii. $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ spermine

(e) Aromatic amine:



benzylamine

Figure 1.1. Structures of groups associated with amine oxidases.
(a) Prosthetic group in monoamine oxidase.
(b) Inhibitor for semicarbazide sensitive amine oxidases.
(c-e) Some substrates for amine oxidases.

norepinephrine, dopamine and serotonin, in the central nervous system (8). Its role in psychiatric syndromes and as a target for antidepressant drugs has also been reported (2,9).

MAO is located in the outer membrane of mitochondria of various tissues and it has been isolated and characterized. The two types widely acknowledged are MAO-A and MAO-B, which are distinctly different from the copper-containing amine oxidases.

Semicarbazide-sensitive amine oxidase (SSAO)

Although this group of enzymes has not been well studied, the semicarbazide-sensitive amine oxidases are widely distributed in vascular and adipose tissues of animals, and form a substantial but diffuse group of enzymes. For example, the Cu-AO's are sensitive to semicarbazide (Fig. 1.1) (2), but this sensitivity is relatively low when compared to that of brown adipose tissue or aorta of the rat, two tissues very rich in SSAO (4). The nature of the cofactor in these very sensitive SSAO's is unknown. Moreover, attempts at demonstrating the presence of copper in the brown adipose tissue enzyme have so far failed. However, kinetic distinctions (4) as well as unique stereochemical patterns (Scaman, C.H., unpublished results) separate this group of SSAO from the flavin- and copper-containing amine oxidases.

Copper-containing amine oxidases

The copper-containing amine oxidases (Cu-AO's) (EC. 1.4.3.6) catalyze the oxidative deamination of mono-, di- and polyamines, forming stoichiometric amounts of aldehydes, hydrogen peroxide and ammonia, as was shown in equation 1.

The physiological role of these amine oxidases is unknown. However, they have been implicated in growth and differentiation, e.g. spermine as growth factor for micro-organisms and its oxidized product as a bifunctional cross-linking agent for stabilizing complementary strands of DNA. As well, perturbation in the enzyme has been associated with certain pathological conditions such as cancer, uremia (latter stages of renal failure), behaviour disorders, and pregnancy (2,7).

These enzymes have been found in eukaryotic cells of fungal, plant and mammalian origin (2), and have some common physico-chemical properties. The amine oxidases so far examined are all dimers, made up of two apparently identical subunits containing two copper (Cu^{2+}) atoms per mole of enzyme, an additional organic cofactor, and a fairly constant carbohydrate content of 7-10% for plasma enzymes (10,11) and 12-14% for plant enzymes (12).

The approximate molecular weights of copper-containing amine oxidases range from 170,000-190,000 for bovine plasma amine oxidase (BPAO) and pig kidney diamine oxidase, 186,000-196,000 for porcine serum amine oxidase (PPAO), 180,000-185,000 for pea seedling amine oxidase (PSAO), and 252,000-273,000 for *Aspergillus niger* amine oxidase (12-15). Although the amino acid compositions show no similarities in the plant enzymes (12,13), a similarity seems to exist for the plasma enzymes of bovine and porcine origin (10,11). The gene encoding amine oxidase has been isolated and cloned from the yeast *Hansenula polymorpha* (16). The gene contained 692 amino acids which compared favourably with the amino acid composition when the purified enzyme protein was acid-hydrolyzed and analyzed. The calculated molecular mass of 77,435 Da also compared reasonably well with the apparent molecular mass of 79 kDA determined from SDS-PAGE of the purified amine oxidase (16).

Most reports demonstrating the presence of the 2 copper atoms per mole of enzyme also indicate that the metal atoms are firmly bound in the enzyme molecule (2,17,18). However, there have been claims of removal of at least one copper atom from some of these enzymes by dialysis against cyanide (19,20) or by dialysis against diethyldithiocarbamate (17,21), with subsequent loss in enzyme activity. Reactivation of this catalytically inactive enzyme has also been reported (19,20), by the addition of suitable amounts of Cu^{2+} or by dialysis against cupric copper.

The real identity of the organic prosthetic group in the Cu-AO's has eluded researchers for years. Several studies had demonstrated the presence of a reactive carbonyl

group (2,22-24), tentatively identifying it as a pyridoxal derivative. However, this structure was never confirmed by studies carried out later (25,26). Two independent studies in 1984 (27,28) reported that bovine plasma amine oxidase contained pyrroloquinoline quinone (PQQ). This led to a proliferation of research papers reporting the presence of PQQ in several enzymes of higher organisms (29,30). However, the lack of direct evidence specifying PQQ at the active site of these enzymes of higher organisms has led to further structural research (31).

Although studies after 1984 have eliminated PLP as the cofactor, recent reports still contend that PLP is present in pig plasma amine oxidase (32,33). The most recent study (31), reporting the isolation of the cofactor-containing pentapeptide, followed by a series of structural characterizations, has provided evidence that 6-hydroxydopa (3,4,6-trihydroxy-phenylalanine, topa) and not PQQ is the active site carbonyl cofactor of bovine plasma amine oxidase. Preliminary and unpublished results from the same group of researchers suggest that topa is also present in pig plasma and pea seedling amine oxidase. Figure 1.2 shows the three reactive carbonyl groups which have so far been implicated as the organic cofactor in Cu-AO's.

Most, if not all, of the Cu-AO's isolated and purified have a distinct intense pink colouration which had always been attributed to the organic cofactor and which corresponded to an absorbance in the visible region around 470-500 nm (7).

Amine oxidase was isolated from plant extracts as far back as 1948 (2,10). The Cu-AO's have mostly been found in leguminous plants. The pea seedling amine oxidase (PSAO) is the most studied. The enzyme is absent in ungerminated seeds, but its activity increases during the early stages of germination in the dark. The enzyme activity has been found to be greatest in the growing regions, i.e. embryo, cotyledons, the initial stem of the seedlings, and decreases with maturity and senescence of the plant. The enzyme is active towards the primary amino group of diamines, such as putrescine and cadavarine, and

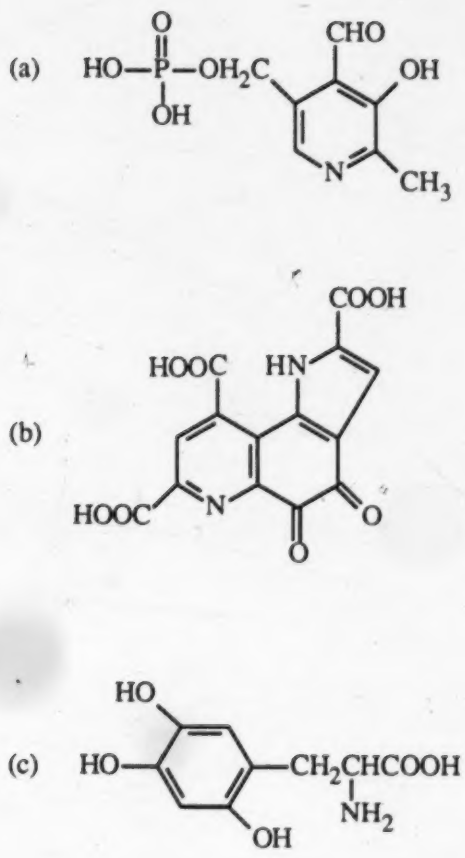


Figure 1.2. Carbonyl cofactors in amine oxidase.
(a) Pyridoxal-5'-phosphate (PLP)
(b) Pyrroloquinoline quinone (PQQ)
(c) 3,4,6-trihydroxyphenylalanine (TOPA)

towards the secondary amino groups of the polyamines spermine and spermidine (Fig. 1.1) (2,12).

In animals, the enzyme is present in and can be isolated from the plasma, e.g. in bovine, porcine, sheep, rabbit, goat, horse, camel, dog, elephant and human. It is also present in tissues and organs such as connective tissues (as lysyl oxidase involved in the cross-linking of collagen) and in pig kidney (pig kidney diamine oxidase) (1,2,7).

The isolation of sheep (34) and bovine (35) amine oxidases in 1953 and 1954 paved the way for the distinction of these enzymes in mammalian plasma. Sheep and cattle are ruminants, and these animals were found to have a characteristic amine oxidase which was very active towards the substrates spermine and spermidine. These substrates are polyamines containing both primary and secondary amino groups (Fig. 1.1). This enzyme was found to resemble monoamine oxidase in its substrate specificity (active towards monoamines) and histaminase (diamine oxidase) in its inhibitor specificity (1). Because of its higher activity towards spermine and spermidine than benzylamine (Fig. 1.1), it became known as spermine oxidase. This characterization distinguished it from benzylamine oxidase, which was found initially in horse serum (36). Although benzylamine oxidase also resembled monoamine oxidase in its substrate specifity and histaminase in its inhibitor specificity, it was without any significant activity towards spermine or spermidine. Its rather high activity towards benzylamine led to its name. The original difference reported between these two enzymes was that benzylamine oxidase did not have any significant action on spermine or spermidine, whereas spermine oxidase oxidized these two polyamines preferentially. In addition, the spermine oxidase was reactive towards all the amines which were substrates of benzylamine oxidase, including benzylamine (37). The isolation of benzylamine oxidase from pig plasma has made it a convenient source of purification (1,38). Blaschko and Hawes observed in 1959 (39) that spermine oxidase was associated with all ruminants and some non-ruminants which had evolved special compartments for fermentation of cellulose, e.g. hippopotamus, and benzylamine with the

rest of the non-ruminants. Studies on the origin of the enzyme led to the conclusion that spermine oxidase arose in phylogenesis, alongside the evolution of the rumen, from an amine oxidase (benzylamine oxidase?) which did not have any significant action on spermine (39).

The true substrate for benzylamine oxidase is not yet known, however the high activity of the enzyme towards benzylamine has made researchers speculate that the substrate for this enzyme is closely associated with, or similar to, benzylamine which is physiologically insignificant (4).

Stereochemical studies on Cu-AO have revealed some unusual patterns in the oxidation of some chiral primary aromatic and aliphatic amines (see Appendix 1 for stereochemical nomenclature used in text). Battersby and his coworkers reported in 1976 and 1979 that the pea seedling amine oxidase (PSAO) oxidized benzylamine and dopamine in a stereospecific fashion by abstracting the pro-*S* hydrogen at C-1 of the substrates (40,41). Following these initial studies, the reaction of the bovine plasma enzyme (BPAO) was also researched. Suva and Abeles in 1978, and Battersby *et al.* in 1979 both observed that BPAO also catalyzed the abstraction of the pro-*S* hydrogen from *p*-hydroxybenzylamine and benzylamine, respectively (25,42). It also abstracted the pro-*S* hydrogen from 3-methylbutylamine (43). However, when studies by Summers *et al.* in 1979 were extended to include dopamine as substrate, they found that BPAO's abstraction of the C-1 hydrogen was nonstereospecific, and they proposed that dopamine had two binding modes, characterized by absolute but opposing stereochemistries (44). These results were confirmed in 1986 by Farnum and Klinman (45).

In 1989, Coleman *et al.*, studying the stereochemical course of the porcine plasma enzyme reaction, reported yet another different stereochemistry for a Cu-AO. It was observed that PPAO catalyzed dopamine and tyramine stereospecifically, however the reaction proceeded with pro-*R* abstraction of the C-1 hydrogens of either substrate (46). In this same study, results of the previous experiments by Battersby *et al.* (1979), Summers *et*

al. (1979) and Farnum and Klinman (1986) were confirmed using deuterated substrates rather than tritiated ones, and analyzing the results by ^{1}H NMR (nuclear magnetic resonance). Reactions involving tyramine with PSAO and BPAO also showed pro-*S* specificity and nonstereospecificity, respectively.

These results provided the first example known in which a reaction catalyzed by enzymes in the same formal class (Cu-AO) occurred with the three possible stereochemical routes (i.e. pro-*S* and pro-*R* specificity and nonstereospecificity).

Lovenberg and Beaven in 1971 were able to differentiate the copper- from the flavin-containing amine oxidase by studying the solvent exchange properties of BPAO and liver mitochondrial amine oxidase with C-2 tritiated dopamine as substrate. They observed a 50% reduction in label at C-2 of the reaction products when BPAO was the enzyme used in reaction as against a complete retention of label when liver mitochondrial amine oxidase was used (47). It is now known that not all Cu-AO's have this solvent exchange property (46).

The pro-*R* and nonstereospecific enzymes exchange solvent (label) into C-2 of product from dopamine or tyramine reaction, while the pro-*S* enzymes do not. This observation has been confirmed recently in studies using five other Cu-AO's, namely rabbit and sheep plasma, porcine kidney, soybean and chick pea amine oxidases. The plasma enzymes were all nonstereospecific in abstracting the C-1 hydrogen of tyramine and exchanged solvent into the C-2 position of product, whereas the plant and kidney enzymes, which were pro-*S* at C-1, did not incorporate label into C-2 of reaction products (48). Studies conducted in 1986 have reported that the C-1 and C-2 hydrogens of dopamine are removed in a syn fashion at the active site of the bovine enzyme. This observation thus supports the presence of a single base at the active site of bovine plasma amine oxidase (45).

The stereochemistry of porcine plasma amine oxidase reaction at C-1 and C-2 of tyramine and dopamine is the major subject of this thesis, which is presented in a manuscript format. The topics for the subsequent chapters deal with:

(1) Purification and isolation of the enzymes used in the study.

(2) Stereochemical course of oxidation at C-1 of tyramine and dopamine, and the solvent exchange characteristics of porcine, bovine, rabbit and pea seedling amine oxidases.

(3) Stereochemical course of oxidation at C-2 of tyramine and the stereochemical relationship between C-1 and C-2 proton abstraction of the same substrate with bovine and porcine plasma amine oxidases.

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2. Isolation and Purification of Amine Oxidases

Introduction

Recent interest in copper amine oxidases led to the search and identification of some of these extramitochondrial enzymes from several animal and plant sources. These enzymes catalyze the oxidative deamination of benzylamine very rapidly, even though the true physiological substrate of the mammalian enzyme is unknown (1). Reports have associated vascular changes with copper-deficient animals which show abnormal amine oxidase levels, yet the physiological significance of these enzymes is still unclear. Enzymes from plant sources have also been associated with regulation of cellular amine levels. The initial interest in isolating this enzyme from rabbit plasma was to help in identifying a source from a laboratory animal, to facilitate investigations into the physiological roles of the enzyme (2).

The porcine (PPAO) and rabbit (RPAO) plasma amine oxidases were isolated to elucidate the stereochemical course of proton abstraction at C-1 of substrates, as well as stereochemical studies at C-2 of substrates. The pea seedling enzyme (PSAO) and the bovine plasma enzyme (BPAO) had previously been isolated and their stereochemistries were known (3-5). However, the pea seedling enzyme was isolated here with the aim of confirming the previous stereochemistry. This report presents the purification procedures used for isolating all of these enzymes and describes some characteristics of the enzymes observed from gel electrophoresis.

Materials

Fresh porcine blood was purchased from Gainers (Edmonton, AB), frozen young rabbit plasma from Pel-Freeze Biologicals (Rogers, AR), and Laxton's Progress garden peas (*Pisum sativum*) from a local food store. Benzylamine hydrochloride and herring protamine sulfate were purchased from Sigma Chem. Co. (St. Louis, MO). Other chemicals were of reagent grade and were used without further purification.

Bio-Gel A1.5m, hydroxyapatite, Bio-Rad Protein Kit and Electrophoresis Kit and reagents were purchased from Bio-Rad Laboratories (Mississauga, ON). DEAE-cellulose (DE-52) and phosphocellulose (P-11) were from Whatman Co. (Maidstone, England), and Con A-Sepharose was purchased from Sigma Chem. Co. (St. Louis, MO).

Methods

Purification of porcine plasma amine oxidase (PPAO)

The enzyme was isolated from 15 l of fresh citrated pig's blood by the method described by Falk *et al.* in 1983 (6). A 2.2 l volume of citrate solution (8 g citric acid + 22 g sodium citrate dihydrate/l) was used. The isolation was carried out at 4°C and centrifugations were done at 17,700 x g for 20 min at 4°C. The isolation procedure is described below:

The citrated blood was centrifuged and the pellet containing red and white blood cells was discarded. The supernatant plasma retained (7.7 l) was mixed with $(NH_4)_2SO_4$ for a 35% saturation (209 g/l), stirred for 3 h and the precipitate separated and discarded by centrifugation. The resulting supernatant was mixed with 129 g/l $(NH_4)_2SO_4$ to bring the salt concentration to 55% saturation, stirred overnight and then centrifuged, discarding the supernatant. The pellet was dissolved in a minimum volume of 10 mM sodium phosphate buffer, pH 7.0, and dialysed against four changes of 4 l of the same buffer. The dialysate (870 ml) was centrifuged to remove any solid particles before chromatography.

The dialysed enzyme solution was loaded onto a Whatman DE-52 column (9 x 45 cm), previously equilibrated with 10 mM sodium phosphate buffer, pH 7.0. The column was washed with the same buffer until the absorbance at 280 nm was close to baseline, and then the enzyme was eluted in succession with 30 mM and 100 mM sodium phosphate buffers, pH 7.0, at a flow rate of 2 ml/min. Fractions of 22 ml were collected in test tubes. The fractions showing activity in each of the eluates were pooled separately. The rest of the

isolation procedure was carried out separately on the fractions, collecting 22 ml fractions from the chromatography.

Each of the fractions (A = 1.5 l of 30 mM and B = 1.15 l of 100 mM) was loaded onto a Con-A Sepharose column (2.5 x 30 cm), previously equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$. The enzyme was eluted with the same buffer containing 100 mM methyl α-mannopyranoside at a flow rate of 0.98 ml/min. Fractions with enzyme activity were pooled, concentrated by ultrafiltration (Amicon PM-30 membrane) and dialysed in 10 mM sodium phosphate, pH 7.0.

The dialysate (250 ml A or 730 ml B) was loaded onto a Bio-Gel A1.5m gel filtration column (2.5 x 100 cm), previously equilibrated with 10 mM sodium phosphate buffer, pH 7.0. The enzyme was eluted in the same buffer at a flow rate of 0.46 ml/min. The fractions showing the most enzyme activity were pooled, ultrafiltered and dialysed in 6mM sodium phosphate buffer, pH 7.0.

The dialysate (132 ml B) was loaded onto a hydroxyapatite column (2.5 x 30 cm), previously equilibrated with 6 mM sodium phosphate buffer, pH 7.0. The enzyme was eluted in succession with 6 mM and 60 mM sodium phosphate buffers, pH 7.0, at a flow rate of 0.6 ml/min. The most active fractions in each eluate (pale pink in colour) were pooled, concentrated and dialysed against 50 mM sodium phosphate buffer, pH 7.0, and stored at 4°C.

An additional chromatographic step involving an AH-Sepharose column in the purification procedure, introduced by Kang (7), has resulted in an increased specific activity and better yield of the enzyme.

Isolation of pea seedling diamine oxidase (PSAO)

Garden pea seeds (*Pisum sativum*) were soaked in running water for 48 h at room temperature. The pea seeds were then germinated at room temperature for 9 days in the

dark, in containers filled with moist vermiculite. The containers were covered with aluminum foil which was punched in several places to allow air into the containers. The method of isolation was a combination of procedures (8-11).

About 3.5 kg of germinated pea seedlings, freed of roots, were washed and homogenized with cold 50 mM potassium phosphate buffer, pH 6.9, at 4°C. A final ratio of 1:1.5 pea seedlings to buffer was used, and the homogenate was filtered through cheese cloth before centrifugation at 11,300 x g for 40 min at 4°C.

The supernatant was slowly mixed with a 5% solution of protamine sulfate (1 g per 10 g protein), stirred for 30 min, and centrifuged. Protamine sulfate binds to DNA and any other forms of nucleic acid released into the solution after the pea seedlings were homogenized.

Ammonium sulfate was added to the supernatant recovered from the protamine sulfate step to make a 35% saturated solution (209 g/l). After stirring for 3 h, the solution was centrifuged and the pellet was discarded. The supernatant was brought to 65% ammonium sulfate saturation (200 g/l), stirred overnight and centrifuged. The pellet was dissolved in a small amount of 20 mM phosphate buffer, pH 6.4, and dialyzed for 24 h against two changes of the same buffer, 7 l each.

The dialysate was clarified by centrifuging the solution and all precipitated protein was removed. The clarified solution was loaded onto a phosphocellulose (P11) column (2.5 x 30 cm), previously equilibrated with 20 mM potassium phosphate buffer, pH 6.4. The column was washed with the same buffer until the eluate showed no absorbance at 280 nm. The enzyme was eluted with 50 mM phosphate buffer, pH 6.9, at a flow rate of 2 ml/min. A fraction size of 13 ml was collected by the fraction collector. Where overloading of the P11 column occurred, the eluted protein was rechromatographed.

Fractions showing enzyme activity were pooled and ultrafiltered in an Amicon cell using a PM-30 membrane (30,000 molecular weight cut off). The concentrated solution was loaded onto a Whatman DE-52 anion exchange column (2.5 x 30 cm), previously

equilibrated with 50 mM potassium phosphate buffer, pH 6.9, and washed with the same buffer at a flow rate of 2 ml/min. The enzyme protein washed through without adsorbing onto the column. Negatively-charged proteins absorbed to the column, thus separating contaminating proteins from the enzyme solution.

The bright pink enzyme solution which eluted early with the equilibrating buffer was pooled separately from the orange-coloured solution eluting in the latter fractions. Both were concentrated by ultrafiltration. The orange-coloured enzyme solution was rechromatographed on the DE-52 column. More enzyme (bright pink) was recovered.

Partial purification of rabbit plasma amine oxidase (RPAO)

The methods of McEwen (12) and McEwen *et al.* (2) were adapted, with a slight modification. All steps were carried out at 4°C and centrifugation at 17,700 x g for 20 min.

Three liters of frozen citrated young rabbit plasma were thawed for two days in a cold room at 4°C. Ammonium sulfate was added to the thawed plasma to make a 34% saturated solution (200 g/l). After stirring for 3 h, the solution was centrifuged. The supernatant was again mixed with ammonium sulfate to bring the solution to 53% saturation (120 g/l), and stirred overnight. The mixture was centrifuged, supernatant discarded, and pellet dissolved in a volume of distilled water corresponding to 11% of the original plasma volume.

K_2HPO_4 (2 M) was added to the dissolved pellet (10% of the volume of dissolved pellet), bringing the volume to a total of 367 ml. The solution was brought to 10% ammonium sulfate saturation by the addition of the salt (100 g/l) and stirred for 1 h. The precipitate was not discarded after centrifugation, but was dissolved in distilled water. Contrary to the literature method, this precipitate contained high enzyme activity. The supernatant was again stirred with more ammonium sulfate (20 g/l), making a 2% solution, and stirred for 15 min. After centrifuging, the precipitate was dissolved in distilled water. This process was repeated four times. The fourth precipitate did not contain any enzyme

activity, thus it was discarded. The fractions showing enzyme activity were pooled. This pooled fraction was blue in colour due to the presence of ceruloplasmin. The pooled fraction was dialysed against three changes of distilled water, 4 l each, and against 4 l of 10 mM potassium phosphate buffer, pH 7.0, for a fourth change.

Dialysate collected (288 ml) was centrifuged to remove any precipitated protein, then loaded onto a Whatman DE-52 anion exhange column (2.5 x 30 cm), previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0. This step replaced the adsorption onto calcium phosphate gel, which had previously resulted in a disappointing purification of another plasma amine oxidase (7). The enzyme was eluted in succession with 30 mM and 100 mM potassium phosphate buffers, pH 7.0, at a flow rate of 2 ml/min. Fractions of 22 ml were collected, and those showing enzyme activity for both 30 mM and 100 mM buffers were pooled and separately ultrafiltered using a PM 30 membrane in the Amicon cell. The 30 mM concentrated fraction showed an orange-red colour and may still have contained some haemoglobin. The blue colour of the 100 mM fraction was due to ceruloplasmin. The overall yield was very low at this stage, therefore the enzyme was used without further purification for the stereochemical studies.

Native polyacrylamide gel electrophoresis was carried out on the partially purified enzyme preparation after the anion-exchange step, to demonstrate the presence of only one amine oxidase, by staining for activity.

Assay of enzyme activity

The enzyme activity was assayed by the method of Tabor *et al.* (13) at 25°C in 50 mM sodium phosphate buffer, pH 7.2, with 3.33 mM benzylamine in a total reaction volume of 1 ml. Benzylaldehyde produced was measured spectrophotometrically at 250 nm. An extinction coefficient of 11.3 mM^{-1} cm^{-1} was used under these conditions. The amount of enzyme required to catalyze the production of 1 µmol of benzaldehyde per min

under the conditions specified was defined as a unit of activity. Spectral measurements were made on the Hewlett-Packard Diode Array Spectrophotometer.

Protein Assay

The Bio-Rad Protein Assay Kit was used. This is based on the Bradford method using bovine serum albumin as a protein standard (14). Absorbance was measured spectrophotometrically at 595 nm on the Hewlett-Packard Diode Array Spectrophotometer.

Electrophoresis

A Bio-Rad Mini Protean II vertical slab gel electrophoresis cell equipped with 1.5 mm spacers was used. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (15). Native gel slabs were made using Tris-borate buffer at pH 8.3 (16) instead of the Tris-HCl buffer used for SDS-PAGE. Only 11% gels were made, and run at a constant current of 18 ma for 2 h. Coomassie Brilliant Blue R-250 (0.002% in ethanol:acetic acid:water, 10:5:85) was used for staining proteins, and destaining was done in the same solvent without the dye (17). Activity staining for amine oxidase was performed according to the method of Ryder *et al.* (18). Tyramine hydrochloride (3.3 mM) was used as substrate in the reagent, which also contained peroxidase and 3-amino-9-ethylcarbazole. An overlay of the reagent on the gel slab produced a coloured and insoluble oxidized product from the coupled reaction with peroxidase and 3-amino-9-ethylcarbazole.

Results and Discussion

Purification of porcine plasma amine oxidase (PPAO)

The purification of the porcine enzyme is summarized in Table 2.1. A final specific activity of 0.059 unit/mg was obtained, which is less than the 0.11 unit/mg reported by Falk *et al.* (6) and Kang (7). As mentioned under methodology, Kang had included an extra chromatographic step during the isolation, i.e. chromatography on an AH-Sepharose column (7).

It has long been the practice to add citrated solution to fresh blood from which an enzyme protein is to be isolated, to prevent coagulation. The addition of $(NH_4)_2SO_4$ for a percent saturation (e.g. 35-55%), at the initial stage, is done to precipitate and eliminate a lot of the contaminating proteins, namely proteins which precipitate before 35% saturation and those which are soluble beyond 55% $(NH_4)_2SO_4$ saturation. The process relied on the salting-out characteristics of $(NH_4)_2SO_4$ on proteins. Thus water molecules, surrounding the hydrophobic surface of proteins, are attracted to the $(NH_4)_2SO_4$ salt for ionic interactions, leaving the exposed surface of the proteins for hydrophobic interactions with other exposed proteins in solution. These hydrophobic interactions result in coagulation, giving the precipitated proteins, which are then discarded. This step is used most often at the start to remove a great amount of unwanted proteins.

The resolution of two different batches of PPAO on DE-52 resulted in two major peaks with different enzyme activities. This observation had been reported in other studies (1,6). The relative amounts of enzyme eluted by increasing concentrations of buffer solutions are shown in Table 2.2. In each of the two instances where the porcine enzyme was isolated, the fraction eluted by 100 mM phosphate buffer on DE-52 contained most of the activity, which after further purification was finally used for the stereochemical studies. This was not always the case though in the work reported by Falk *et al.* (6). They sometimes observed more activity with the 30 mM eluate and also some activity in the

Table 2.1. Purification of porcine plasma amine oxidase (PPAO).

Purification Step	*Volume (ml)*	*Total Units*	*Specific activity (units/mg)*[a]	*Purification (fold)*	*Yield (%)*
Plasma	7,700	83.9	2.9×10^{-4}	1	100
$(NH_4)_2SO_4$ (35-55%)	870	62.4	1.2×10^{-3}	4.1	74.4
DE-52					
30 mM (A)	1,500	2.9	1.8×10^{-4}	0.7	
100 mM (B)	1,150	26.5	3.7×10^{-3}	12.8	31.6
Con A					
Sepharose A	250	1.0	6.3×10^{-3}	21.7	
B	730	19.2	2.9×10^{-2}	100	57
Bio-Gel (A1.5m)					
B	132	14.4	3.2×10^{-2}	110	17.2
Hydroxyapatite					
B1	12	1.45	6.0×10^{-2}	207	1.7
*B2	18	7.4	5.7×10^{-2}	197	8.8

[a] 1 unit = 1 μmol benzaldehyde/min at 25°C
* enzyme used for stereochemical studies

Table 2.2. Purification of porcine blood, showing relative amounts of enzyme activity eluted with different buffer concentrations on DE-52.

	Peaks eluted		
Batch no.	*A (30 mM)*	*B (100 mM)*	*C (10 mM)*
1	9.9	90.1	0
2	12.7	87.3	0

initial eluate which washed through with the loading buffer. There are no real explanations for these differences, although the physical well being of the animal may be one factor, while another may be that some partial hydrolysis of the enzyme protein is occurring. The fact that the enzyme adsorbed to the anion-exchange column (DE-52) implied that the protein was basic in nature. Thus at pH 7.0 of the buffer solutions, the anion resin was positively-charged and attracted only PO_4^{2-} ions, which were subsequently exchanged with negatively-charged proteins in the enzyme solution. It is important to note that the pI of the porcine amine oxidase has been established to be between 4.5 and 5.0 (6), such that at pH 7.0 the net protein charge would be negative, effectively adsorbing the enzyme onto the anion-exchange column. A similar pI was reported for bovine plasma amine oxidase (19) and preliminary (unpublished) experiments in our laboratory on sheep plasma amine oxidase have also indicated a similar pI range, hence these amine oxidases are also basic proteins.

The use of Con A-Sepharose column as a step in the purification procedure was to rid the enzyme of other contaminating proteins, especially non-carbohydrate containing proteins, by the selective binding of glycoproteins to the resin. Con A (concanavalin A), a lectin which is basically a protein with carbohydrate binding affinities specific for α-D-mannopyranosyl and α-D-glucopyranosyl residues, was used to bind the enzyme proteins and other glycoproteins in the enzyme solution. The predominant sugar residue in the porcine plasma amine oxidase has been reported to be mannose (6), hence the effective elution of the enzyme protein with buffer containing 100 mM methyl α-mannopyranoside. The elution profile of the enzyme showed a large peak followed by a long tail, which was suggestive of some variability in the carbohydrate content of the enzyme, or suggestive of the difficulty of the buffer to elute portions of the enzyme which had been adsorbed more strongly to the resin.

Chromatography on the Bio-gel A1.5m gel filtration column relied on separation of proteins according to their sizes. The larger proteins eluted in the void volume faster than

others since they were too large to be entrapped by the gel matrix. The rest of the entrapped proteins then separated from each other (larger ones first) according to the length of time spent in the fraction of pores available for them. Small-sized proteins thus eluted at a much slower rate.

Often used at the last stage of enzyme purifications, hydroxyapatite has been found to resolve enzymes into solutions containing homogenous proteins even though the mechanism involved in the adsorption of proteins to the resin is still unclear. However, hydroxyapatite is reported to be a form of ion exchanger which reacts with proteins by ionic interactions (20). Both the NH_3^+ and COO^- groups on the protein react with PO_4^{3-} and Ca^{2+} on the hydroxyapatite, respectively, resulting in adsorption of the protein onto the resin. Falk *et al.* (6) have reported that the two fractions eluted off the hydroxyapatite column contain the same type of carbohydrates, the only difference being the amounts present in each.

Purification of pea seedling amine oxidase (PSAO)

Table 2.3 summarizes the purification of PSAO. The $(NH_4)_2SO_4$ precipitation process has previously been discussed under the porcine section. As has already been mentioned, protamine sulfate precipitates any nucleic acids and DNA which may have been released during the homogenization of the seedlings. Protamine sulfate, however, does not precipitate proteins, unless the proteins have an affinity for nucleic acids, e.g. ribonuclease.

The chromatography of the pea seedling enzyme on phosphocellulose (P11) relied on the resin for its cationic exchange properties. In addition to this property, P11 also acts as an affinity adsorbent with special affinity for enzymes with phosphate ester substrates (20). PSAO has been shown to have a pI of 7.4 (11), hence at pH 6.4 of the equilibrated column and loading buffer the proteins are positively-charged and adsorb onto the negatively-charged phosphate groups on the resin.

Table 2.3. Purification of pea seedling amine oxidase (PSAO).

Purification Step	*Volume (ml)*	*Total Units*	*Specific activity (units/mg)*[a]	*Purification (fold)*	*Yield (%)*
Crude	5,780	158.6	6.7×10^{-3}	1	100
Protamine Sulfate	5,820	188.6	8.9×10^{-3}	1.3	119
$(NH_4)_2SO_4$ fractionation	985	123.7	1.8×10^{-2}	2.7	78
P11	20	113.8	3.1×10^{-1}	46	72
DE-52	22	80	8.4×10^{-1}	125	50

[a] 1 unit = 1 μmol benzaldehyde/min at 25°C

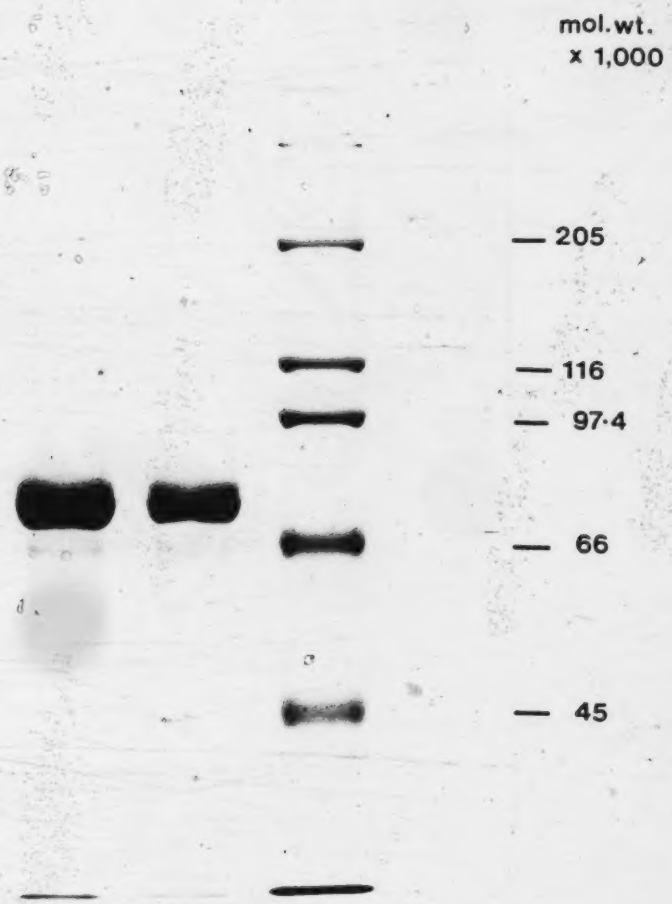

Figure 2.1. SDS-PAGE of pea seedling amine oxidase.

The reverse interaction for porcine enzyme was observed to be the case, however, when pea seedling enzyme was loaded onto the DE-52 anion-exchange column. As a result of its higher pI of 7.4 (11), the enzyme proteins washed through the column with the loading buffer, since the proteins were positively-charged at the equilibrating pH of 6.9.

It was observed that rechromatography of the pea enzyme on DE-52 after storing for about 2-4 weeks in the cold removed additional contaminating proteins. A specific activity of 8.4×10^{-1} (0.84) units/mg was obtained (Table 2.3) with a yield of 50% and purification of 125-fold. The purity is 3x less than was reported by McGowan and Muir (9), while the specific activity is about 84% of their value. The approximate molecular weight of the enzyme subunit from this preparation was estimated from SDS-PAGE to be 87,000, which is in the range of 85,000-92,500 reported in the literature (9-11).

Nine different batches of pea seedling enzyme have been isolated to date in the laboratory, including batches recently isolated by Dr. Palcic. Figure 2.1 is typical for freshly isolated pea seedling enzymes and the molecular weight for this batch was 80,000.

Purification of rabbit plasma amine oxidase (RPAO)

The common purification procedures used for both isolations of the porcine and RPAO, namely citration, $(NH_4)_2SO_4$ precipitation and DE-52 anion-exchange chromatography, have been discussed thoroughly under the section for porcine plasma amine oxidase. Based on the discussion of pI's, one can speculate a similar pI range of 4.5-5.0 for RPAO, which means that RPAO would behave similarly to the porcine enzyme on DE-52. This was found to be the case (Table 2.4).

The purification of rabbit enzyme was rather disappointing (Table 2.4). A purification of about 7-fold, as compared to literature reports (2,12) of 460- and 100-fold, could be attributed to losses incurred during the ammonium sulfate fractionation steps. Whereas greater than 100% yield was reported in each purification step, leading to a final yield of 119% (2), a meagre final yield of 7.8% was obtained in this isolation. The specific

Table 2.4. Purification of rabbit plasma amine oxidase (RPAO).

Purification Step	*Volume (ml)*	*Total Units*	*Specific activity (units/mg)*[a]	*Purification (fold)*	*Yield (%)*
Plasma	3,000	25.5	3.1×10^{-4}	1	100
1st $(NH_4)_2SO_4$ fractionation	367	12.6	1.2×10^{-3}	3.9	49.4
2nd $(NH_4)_2SO_4$ fractionation	288	9.2	1.5×10^{-3}	4.8	36.1
DE-52					
30 mM (A)	11	2.0	2.3×10^{-3}	7.4	7.8
100 mM (B)	18	1.9	1.9×10^{-3}	6.1	7.4

[a] 1 unit = 1 μmol benzaldehyde/min at 25°C

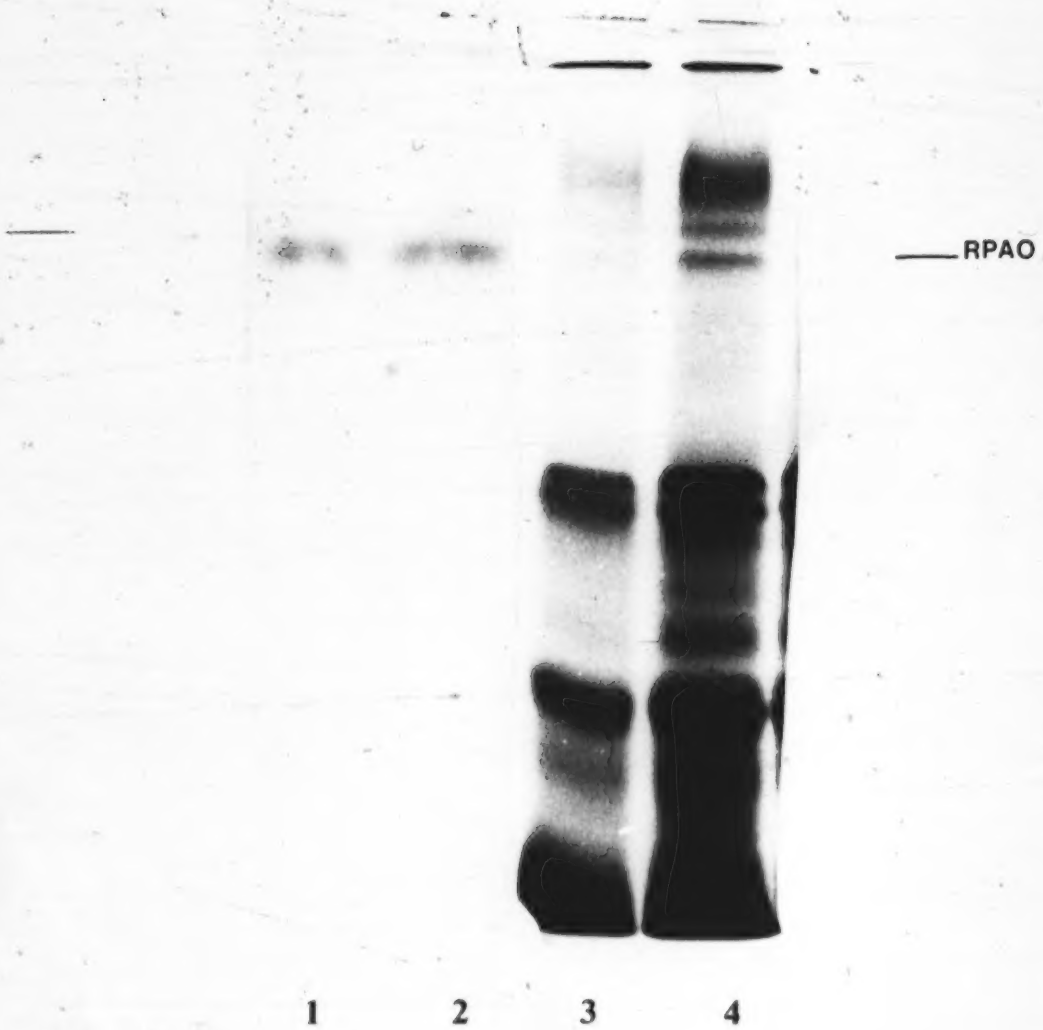

Figure 2.2. Native gel electrophoresis of RPAO
showing: activity stain (lanes 1 & 2)
coomassie blue stain (lanes 3 & 4)

activity of 2.3 x 10^{-3} (0.0023 unit/mg) was 7.5-16x less than has been reported (2,12). McEwen (12) mentioned in his report that further purification of the enzyme (after calcium phosphate gel chromatography) on DEAE cellulose (anion exchange) and Sephadex G-200 (sizing gel) gave a highly-purified enzyme preparation with an activity which will be close to 32x more than was obtained in my isolation. The native gel electrophoresis run on the enzyme isolated indicated that even with 7-fold purification only one major band is present and is responsible for enzyme activity (Figure 2.2).

The native gel stained with Coomassie blue for protein detection (Figure 2.2) gave an estimated molecular weight value for RPAO of around 194,000, a value equivalent to that for porcine enzyme (7). The RPAO protein was run alongside a purified preparation of sheep plasma amine oxidase (SPAO), whose subunit molecular weight had previously been estimated to be approximately 97,000 from SDS gel electrophoresis (7). The RPAO enzyme protein showed a single band on the native gel stained for activity and also corresponded with a specific band on the protein stained gel. Both proteins had migrated in the gel with the same R_f.

In their study, McEwen *et al.* (2) reported that the specific activity of RPAO with benzylamine as substrate is one-sixth that of PPAO (porcine), one-fifth that of BPAO (bovine), and approximately equal to the most purified enzyme preparation of human plasma amine oxidase.

The enzymes isolated, i.e. porcine and rabbit plasma amine oxidases and the pea seedling amine oxidase, have been used in stereochemical studies. The results of these studies are presented in Chapters 3 and 4.

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3. Stereochemistry of Copper Amine Oxidase Reactions[1]

Introduction

Two classes of enzymes catalyze the oxidative deamination of primary amines (Equation 3.1), the flavin-containing monoamine oxidases (E.C. 1.4.3.4), and the copper-containing amine oxidases (E.C. 1.4.3.6) (1).

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2 \qquad [3.1]$$

The established physiological role of the monoamine oxidases in neurotransmitter metabolism has made these enzymes the subject of intensive investigation, notably as targets for antidepressant drugs. Both the biological function and role of copper in the latter enzymes is unclear, and they contain a different organic cofactor, now believed to be TOPA in bovine plasma amine oxidase (2). From 1984 until quite recently, reports had implicated pyrroloquinoline quinone (PQQ) as the organic cofactor present in the copper amine oxidases (3-6). The two enzyme types are also mechanistically distinct as solvent exchange at C-2 of dopamine during catalysis has been observed only with the copper enzymes (7).

Previous stereochemical studies on copper amine oxidases have shown that the pro-*S* hydrogen is lost from C-1 of benzylamine and dopamine when oxidized by the enzyme isolated from pea seedlings (8,9). Bovine plasma amine oxidase also catalyzes abstraction of the pro-*S* hydrogen from benzylamine and *p*-hydroxybenzylamine (10,11). The bovine enzyme, however, exhibits mirror-image binding and processing of dopamine, resulting in nonstereospecific proton abstraction at C-1 and C-2 (12,13). In this chapter we apply ^1H NMR spectroscopy to confirm and extend the stereochemistries deduced previously for the bovine and pea seedling enzymes using radiochemical labeling techniques. In addition, we establish that amine oxidation by a third copper enzyme, porcine plasma amine oxidase,

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1 A version of this chapter has been published: Coleman, A.A., Hindsgaul, O. and Palcic, M.M. (1989). *J. Biol. Chem.*, **264**, 19500-19505.
Coleman, A.A., Scaman, C.H., Kang, Y.J. and Palcic, M.M. (accepted by *J. Biol. Chem.*) — part of the work in this paper is included in this chapter.

occurs with removal of the pro-*R* hydrogen from C-1 of substrate. This represents the first instance where all possible stereochemical reaction courses are followed by enzymes in the same formal class.

Materials

The chemicals used were of reagent grade and were used without further purification. NADH, pyridoxal phosphate, tyrosine, 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenethylamine, tyramine, and benzylamine were from Sigma. Cyclohexanol was obtained from American Chemicals Ltd. and ^2H_2O (99.8% deuterium) from Aldrich. L-[U-^{14}C]tyrosine (450 mCi/mmol) was from ICN and ACS liquid scintillation cocktail was from Amersham. All solvents were reagent grade and were freshly distilled before use.

Methods

Enzymes

Porcine plasma amine oxidase was isolated from 15 l of fresh citrated porcine blood as previously described and outlined in Chapter 2 (14). Isolation steps included $(NH_4)_2SO_4$ fractionation, and sequential column chromatography on DE-52 cellulose, Con A Sepharose, Bio-Gel A1.5m, and hydroxyapatite. The final specific activity of the isolated enzyme was 0.057 unit/mg, when assayed spectrophotometrically at 250 nm, using 3.3 mM benzylamine as a substrate and an extinction coefficient of 11.3 $mM^{-1}cm^{-1}$. Protein concentration was estimated using a Bio-Rad kit which is based on the Bradford method (15) with bovine serum albumin as a reference standard protein. Pea seedling (*Pisum sativum*, cv Laxton's Progress) amine oxidase was isolated from 500 g of 9 day-old etiolated seedlings. Isolation steps included protamine sulfate treatment, $(NH_4)_2SO_4$ fractionation and chromatography on phosphocellulose as outlined in Chapter 2 (16). The final specific activity was 0.051 unit/mg when assayed as above. Frozen rabbit plasma was from Pel-Freez and the enzyme was isolated from 3 l of the frozen citrated plasma. Isolation steps included $(NH_4)_2SO_4$ fractionation (17,18) and chromatography on DE-52

(loaded with 10 mM sodium potassium phosphate buffer, eluted with 30 and 100 mM buffer, all at pH 7.0). The final specific activities of the enzymes were 2.8 x 10^{-3} for the 30 mM eluate and 1.8 x 10^{-3} for the 100 mM eluate. Rabbit plasma enzyme activity was estimated spectrophotometrically with 3.3 mM benzylamine in 200 mM phosphate buffer at pH 7.2 (17,18). Tyrosine decarboxylase apoenzyme, horse liver alcohol dehydrogenase, catalase and bovine plasma amine oxidase were obtained from Sigma.

Synthesis of DL-[2-^{2}H]Amino Acids

This synthesis was carried out by Dr. O. Hindsgaul in the Chemistry Department, University of Alberta. 3'-Methoxytyrosine was reacted with KO^{2}H in ^2H_2O, in the presence of pyridoxal hydrochloride, using the general procedure described by Fujihara and Schowen (19) for α-deuteration of amino acids. The product 3'-methoxy-DL-[2-^{2}H]tyrosine was obtained as a solid after neutralization of the reaction mixture and was recrystallized from hot water by addition of ethanol. DL-[2-^{2}H]Tyrosine and DL-[2-^{2}H]phenylalanine were similarly prepared. The isolated yields were in the range 20-30% and ^{1}H NMR spectroscopy showed over 98% deuterium incorporation at C-2 of all three amino acids. Treatment of 3'-methoxy-DL-[2-^{2}H]tyrosine with HBr (20) gave DL-[2-^{2}H]dopa.

Synthesis of Chiral Amines

Tyrosine decarboxylase apoenzyme was extracted from 150 mg (90 units) *Streptococcus faecalis* cells, into 20 ml of 50 mM sodium acetate buffer pH 5.7 containing 0.45 mM pyridoxal phosphate by grinding with alumina or pulsed sonication for 8 min. Following centrifugation, the supernatant was concentrated to 1 ml by ultrafiltration, resuspended in 80 ml of 50 mM sodium acetate buffer, pH 5.7, and concentrated to 1 ml to remove excess pyridoxal phosphate. This was resuspended in 80 ml of buffer and reconcentrated to 5 ml. Extracted enzyme was assayed, by Dr. M. Palcic, by measuring $^{14}CO_2$ evolution from radiolabeled tyrosine. Incubation mixtures contained 1.3 mM L-

tyrosine, 0.16 μCi L-[U-^{14}C]tyrosine, 0.45 mM PLP and 5 μl extract in 0.5 ml 50 mM sodium acetate buffer, pH 5.7. Reactions were carried out in scintillation vials with a piece of filter paper impregnated with 20 μl of 1 M hyamine hydroxide taped in the vial lids. Following incubation at 37°C for 10 min, mixtures were quenched with the addition of 0.2 ml of 10% trichloroacetic acid, and shaken for 1 h to ensure that evolution of $^{14}CO_2$ was complete. The filter papers were removed and counted in 10 ml of ACS cocktail. The enzyme was extracted at 20-30% yield and had a specific activity of 1-5 units/mg.

For (1*S*)-[^{2}H]amine synthesis, incubations contained 50 mg of the appropriate DL-[2–^{2}H]amino acid and 5 units of tyrosine decarboxylase extract in 10 ml 50 mM sodium acetate buffer, pH 5.7. After 24 h reaction at ambient temperature, the mixture was applied to a column (2 x 5 cm) of Amberlite IRC 50 (H^+ form) equilibrated with water. Unreacted amino acid was eluted with water, then amine eluted with 1 M acetic acid. Fractions were pooled and evaporated to dryness under reduced pressure, after the addition of 0.2 N HCl. (1*R*)-[^{2}H]amines were synthesized in an analogous fashion starting with unlabeled amino acids. In this case incubations were carried out in 10 ml of 50 mM ^{2}H-sodium acetate buffer, pD = 6.4, where pD is 0.4 + the uncorrected pH meter reading.

Stereochemistry of Amine Oxidase Reactions

Incubations were carried out in a coupled fashion (7,11) or a modification of the method by Battersby *et al.* (9), and contained in 1.0 ml:15 μmol stereospecifically deuterated amine, 0.3 unit amine oxidase, 11,000 units catalase, 3 units horse liver alcohol dehydrogenase, 1.1 μmol NADH, 15 μmol cyclohexanol and 100 μmol sodium or potassium phosphate buffer, pH 7.0. Following incubation for 16 h at ambient temperature, samples were diluted to 10 ml with water and loaded onto reverse-phase C-18 Sep-Pak cartridges. The cartridges were rinsed with 5 ml water, then alcohol product was eluted with 10 ml methanol or the product was extracted into ethyl acetate. The methanol and ethyl acetate eluates were concentrated by evaporation under reduced pressure, then

applied to preparative silica gel TLC plates or chromatographed on silica gel by flash chromatography and developed with ethyl acetate/hexane (2:1). (R_f = 0.29 for 3,4-dihydroxyphenethyl alcohol, R_f = 0.51 for *p*-hydroxyphenethyl alcohol). All stereochemical studies were done at least in duplicate.

Solvent Exchange into Product

To monitor solvent exchange into alcohols, incubations were carried out as described for the stereochemical studies with chiral amines, except reactions were carried out in 100 mM deuterated sodium or potassium phosphate buffer, pD 6.9, containing 15 μmol of unlabeled dopamine or tyramine.

Solvent Exchange into Dopamine

Incubations were carried out as for the solvent exchange into alcohols, in 100 mM deuterated sodium or potassium phosphate buffer, pD, 6.9, for 4 hr. The extent of reaction was estimated by separation of starting dopamine from product 3,4-dihydroxyphenethyl alcohol on ion-exchange Accell CM-cartridges and quantitation by measuring absorbance at 280 nm.

^{1}H NMR Spectroscopy

^{1}H NMR spectra were measured at 360 MHz on a Bruker WM-360 instrument operating at ambient temperature (22 ± 1°C). Spectra of amine hydrochloride salts were recorded in ^2H_2O with the residual H^2HO signal (4.81 ppm) used as internal reference standard. Spectra of alcohols were recorded using 4:1 $(^2H_3C)_2CO$:^2H_2O as solvent with the residual H^2H_2C signal (2.19 ppm) as internal reference standard. The absolute chemical shifts using these solvent peaks as reference standards were reproducible to better than 0.05 ppm. Spectra were accumulated into 16 K of computer memory using a 45° pulse with 7 s delays between pulses. Longer relaxation delays did not measurably (<< 5%) affect the relative intensities of the signals as measured by integration.

Results and Discussion

Tyrosine decarboxylase, a pyridoxal phosphate containing enzyme, was used to synthesize stereospecifically deuterated amines. Reactions catalyzed by this enzyme have been well studied, and the enzyme has been shown to operate stereospecifically such that the replacement of the carboxyl group with a solvent proton occurs with retention of configuration (21). Since the enzyme decarboxylates only L-amino acids, unresolved mixtures of α-deuterated amino acids can be used as starting materials. In addition, tyrosine decarboxylase exhibits broad substrate specificity and can be employed for the synthesis of stereospecifically labelled dopamine and phenethylamine as well as tyramine.

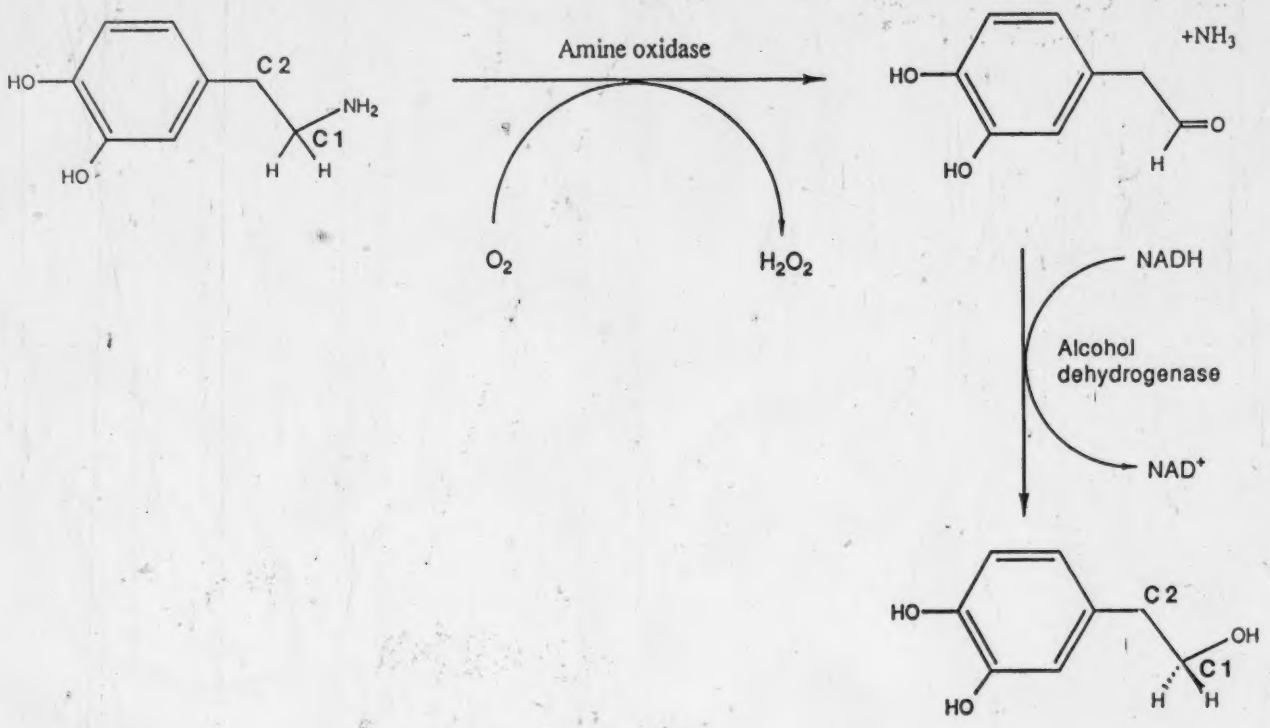
Fig. 3.1 shows the ^1H NMR spectra of stereospecifically deuterated dopamines isolated after incubation of L-dopa in ^2H_2O and DL-[2-^{2}H]dopa in H_2O respectively, with tyrosine decarboxylase. The deuteration can be seen to be complete within the limits of sensitivity of the ^1H NMR spectra (≥98%) and the absolute configuration of the chiral dopamines are assigned based on the established stereospecificity of the tyrosine decarboxylase reaction. Caution must be exercised in prolonged incubations of aromatic amino acids with excess pyridoxal phosphate added as an enzyme cofactor, since nonenzymatic exchange of the α-protons by the cofactor alone can result in scrambling in the final amine products.

Incubations of the chiral amines with the four amine oxidases were carried out in a coupled fashion, such that the aldehyde products generated were converted directly into alcohols (Scheme 3.1). The ^1H NMR spectra of 3,4-dihydroxyphenethyl alcohols isolated from incubations with (1*R*)-[^{2}H]dopamine with porcine, pea seedling, and bovine plasma amine oxidase are shown in Figs. 3.2, 3.3 and 3.4. Results for the stereochemical studies are summarized in Table 3.1. The alcohol obtained from incubation with porcine plasma amine oxidase (Fig. 3.2a) exhibits triplets at 3.73 ppm (C-1 proton signals) and at 2.74 ppm (C-2 proton signals), a pattern requiring full protonation at both positions. Loss of

Table 3.1. Characteristics of enzymatic products from C-1 oxidation of substrates as analyzed by ^1H NMR*.

Enzyme / Stereochemistry	*Substrate*	*Alcohols: monodeuterated (% ^2H retained)*	*diprotonated (% loss)*	*KIE*	*Wash-in at C-2 (# ^2H atoms)*
Porcine -*R*	(1*R*)-dopamine	0	100		
	(1*S*)-dopamine	>95	0		1.2 ± 0.05
Pea Seedling -*S*	(1*R*)-dopamine	>95	0		
	(1*S*)-dopamine	0	100		0
Bovine Nonstereospecific	(1*R*)-dopamine	85	15	5.6	
	(1*S*)-dopamine	94	6	15.6	
	(1*R*)-tyramine	82	18	4.5	
	(1*S*)-tyramine	87	13	6.7	
	(1*R*)-tyramine (4°C)	80	20	4.0	
	(1*S*)-tyramine (4°C)	93	7	13.3	0.8-1.1
Rabbit Nonstereospecific	(1*R*)-tyramine	73	27	2.7	
	(1*S*)-tyramine	82	18	4.6	0.7 ± 0.04

* experiments were done at least in duplicates



Scheme 3.1. Synthesis of 3,4-dihydroxyphenethyl alcohol from dopamine in the coupled enzyme reaction system.

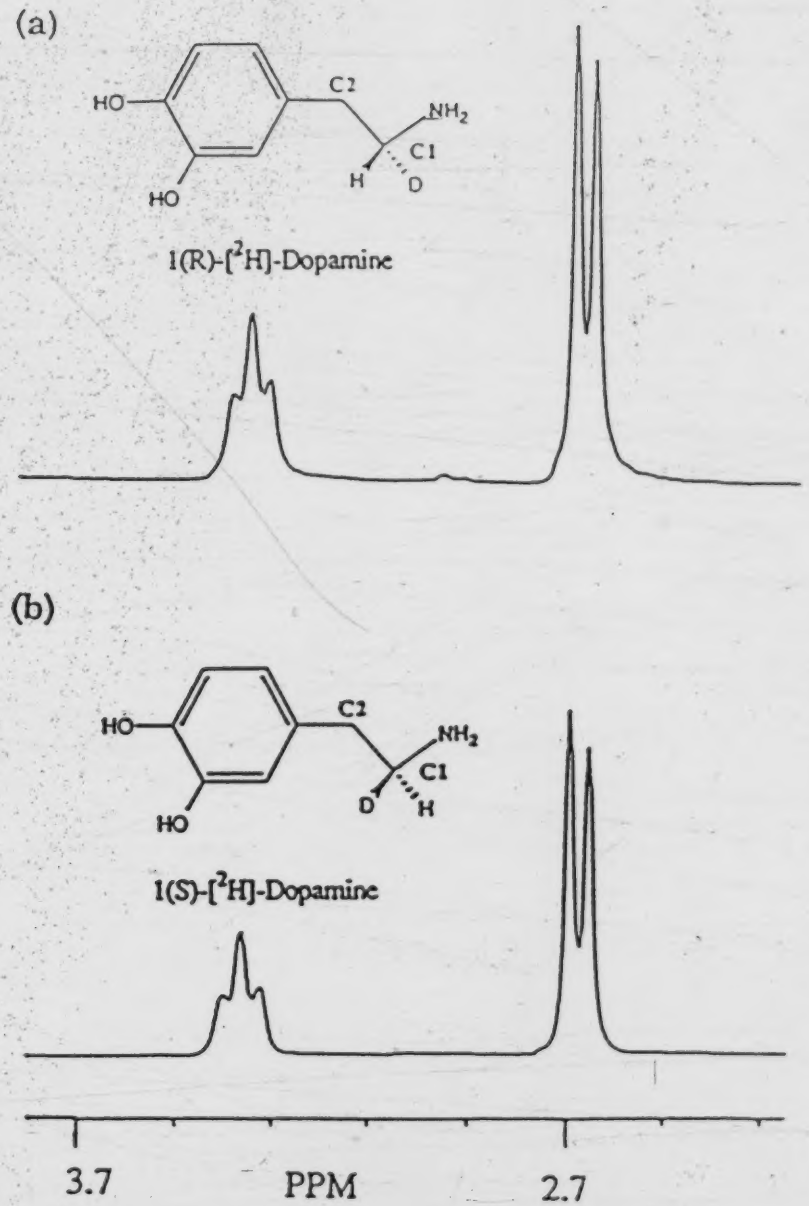


Figure 3.1. ^{1}H **NMR spectra of (1*R*)- and (1*S*)-[^{2}H]dopamines derived from incubations of tyrosine decarboxylase with L-dopa or α-deuterated DL-dopa in (a) $^{2}H_2O$ and (b) H_2O.**

Figure 3.2. ^{1}H NMR spectra of alcohols derived from coupled incubations of porcine amine oxidase and stereospecifically labelled dopamines.
(a) (1*R*)-[^{2}H]dopamine
(b) (1*S*)-[^{2}H]dopamine

Figure 3.3. ^{1}H NMR spectra of alcohols derived from coupled incubations of pea seedling amine oxidase and stereospecifically labelled dopamines.
(a) (1*R*)-[^{2}H]dopamine
(b) (1*S*)-[^{2}H]dopamine

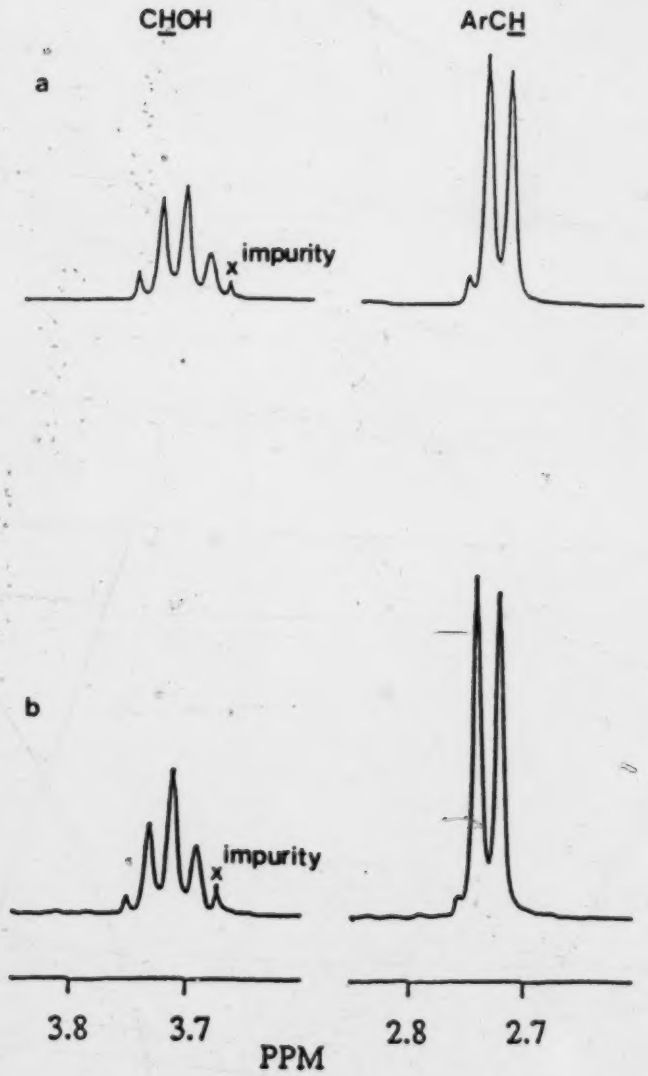


Figure 3.4. ^{1}H NMR spectra of alcohols derived from coupled incubations of bovine plasma amine oxidase and stereospecifically labelled dopamines.
(a) (1*R*)-[^{2}H]dopamine
(b) (1*S*)-[^{2}H]dopamine

deuterium from (1*R*)-[^{2}H]dopamine during reaction thus establishes that dopamine oxidation occurs with loss of the pro-*R* hydrogen (or deuterium in the (*R*)-labeled compound), at C-1 during oxidation. The alcohol sample isolated from an incubation containing pea seedling enzyme and (1*R*) dopamine contained deuterium (Fig 3.3a), confirming the pro-*S* hydrogen abstraction previously reported for this enzyme (7,8). The clean doublet at 2.72 ppm attests to the complete retention of deuterium in this alcohol sample. We attribute the large downfield signal near 3.73 ppm to an impurity in the incubation mixture. The 3,4-dihydroxyphenethyl alcohol isolated from incubations with the bovine amine oxidase, yields a more complex spectrum (Fig. 3.4a). The complex pattern near 3.7 ppm is attributed to two overlapping triplets for C-1. The minor triplet at 3.73 ppm is assigned to the product alcohol diprotonated at C-1. The major triplet at 3.71 ppm arises from 3,4-dihydroxyphenethyl alcohol, mono-deuterated at C-1, which is approximately 0.02 ppm shifted to higher field due to an α-deuterium isotope effect (22). The corresponding signals for the C-2 protons are seen at 2.74 ppm (triplet) for the minor diprotio-alcohol and an upfield β-deuterium isotope shifted doublet at 2.72 ppm for the major monodeutero product. The relative intensities of these NMR signals indicates that 85% of the original deuterium of (*R*)-dopamine is retained (Table 3.1). Appendix 2 shows how the deuterium content in the alcohol products was estimated.

In complementary experiments with (1*S*)-[^{2}H]dopamine, deuterium was retained (>95%) in the 3,4-dihydroxyphenethy alcohol isolated from incubations with porcine plasma amine oxidase, as predicted for a reaction with abstraction of the pro-*R* hydrogen (Fig. 3.2b). Samples derived from reaction of pea seedling amine oxidase, were devoid of deuterium (Fig. 3.3b), establishing the loss of the pro-*S* hydrogen during catalysis. The alcohols obtained from incubations with bovine amine oxidase again show a complex spectrum, (Fig. 3.4b), overlapping triplets near 3.7 ppm, and a doublet superimposed on a triplet near 2.7 ppm. These signals are assigned as for (*R*)-dopamine, above (Fig. 3.4a), and their relative intensities indicate that 94% (Table 3.1) of the deuterium was retained,

again demonstrating a partially nonstereospecific reaction course. For a completely non-stereospecific reaction, one would predict a 1:1 mixture of mono-deuterated and fully protonated alcohols. Bovine plasma amine oxidase, however, is known to exhibit a kinetic deuterium isotope effect of 6 for dopamine oxidation (20), resulting in the retention of a preponderance of deuterium in both alcohols derived from chiral substrates. The preferred reaction pathway arises from a kinetic isotope effect (KIE) where the isotope effect results in a reduction in the rate of C-D bond cleavage relative to that of the C-H bond. Thus the KIE of 6 implies that the rate of reaction is 6-fold greater for the C-H bond cleavage than for the C-D bond cleavage which gives products that are enriched with deuterium, i.e. 86% deuterated and 14% protonated alcohols. Our results are consistent with the existence of dual catalytically competent binding modes for dopamine in the active site of bovine plasma amine oxidase as proposed in previous stereochemical studies (12,13). We account for deviations from the ≈ 85:15 ratio predicted for a kinetic isotope effect of 6 (Table 3.1), to unequal flux through the two binding modes, with preferential processing of dopamine through the *R* mode, i.e. abstraction of the pro-*R* proton. This in turn is proposed to arise from different overall primary kinetic isotope effects in each mode, 6 for *R* substrates and 16 for *S*-substrates. Since the K_m for dopamine is 40 μM (20), the reaction changes from saturating V conditions (saturated substrate levels, 10 x K_m) to V/K control (subsaturating substrate concentrations) as substrates become depleted in our stereochemical study. Any isotope effects estimated from our final product ratios will reflect isotope effects on both of these catalytic parameters. Our product ratios are, however, in general agreement with the predicted ratios of 75:25 for *R* dopamine and 90:10 for *S* dopamine extrapolated from primary tritium V/K isotope effects (12,13).

In order to further probe the unusual lack of overall stereospecificity in the bovine plasma amine oxidase reaction, the stereochemical course of the oxidation of tyramine was also investigated. Fig. 3.5a shows the spectrum for *p*-hydroxyphenethyl alcohol derived from 1*R*-[^{2}H]tyramine and bovine plasma amine oxidase. The retention of 82% deuterium

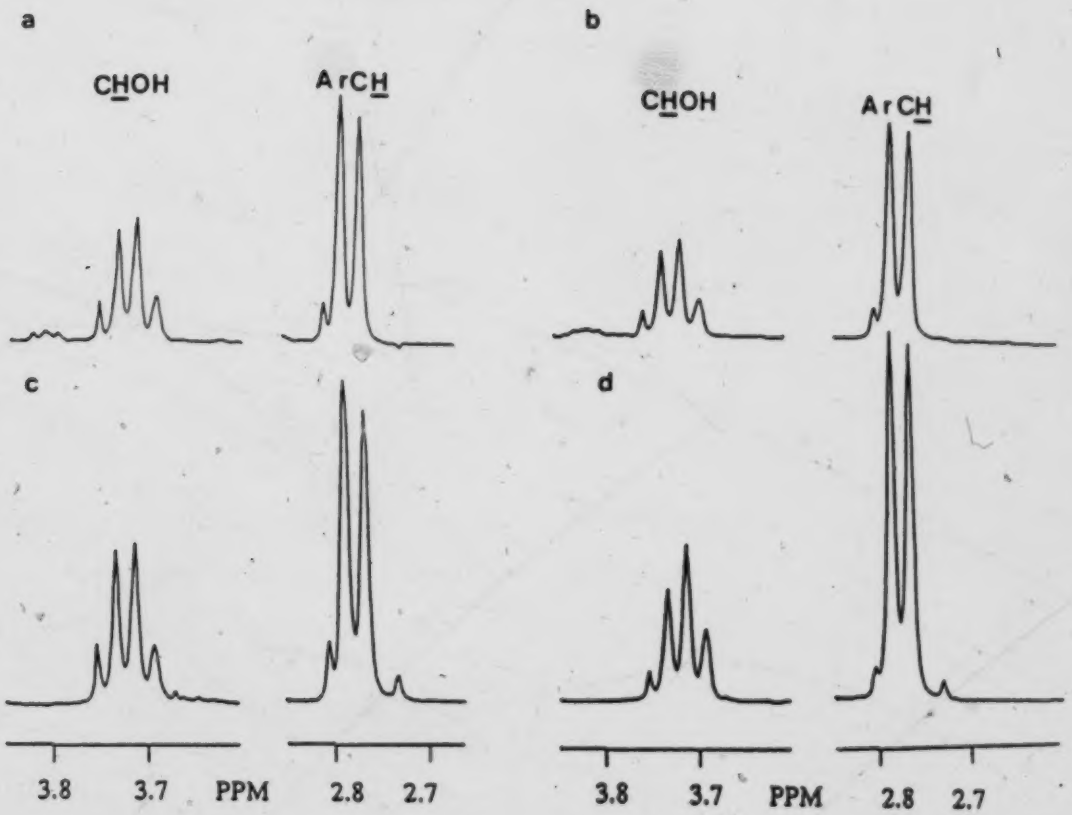


Figure 3.5. ^{1}H NMR spectra of alcohols derived from coupled incubations of bovine plasma amine oxidase and stereospecifically labelled tyramines.

(a) (1*R*)-[^{2}H]tyramine, ambient temperature
(b) (1*S*)-[^{2}H]tyramine, ambient temperature
(c) (1*R*)-[^{2}H]tyramine, 4°C
(d) (1*S*)-[^{2}H]tyramine, 4°C

(and loss of 18%), establishes a nonstereospecific reaction course for tyramine (Table 3.1). This is confirmed in Fig. 3.5b, for the alcohol derived from the 1*S* isomer, which contained a mixture of 87% deuterated and 13% fully protonated molecules. Thus tyramine is also subject to mirror-image binding and processing by bovine plasma amine oxidase. The average measured primary deuterium isotope effect for tyramine oxidation of 6 (20), accounts for the net retention of deuterium in both samples. Tyramine has been shown to exhibit biphasic steady-state plots, yielding K_m's of 1.3 and 52 mM. Our stereochemical experiments are under V/K control and likely not as sensitive to differential isotope effects as the reaction progresses. As with dopamine, the increased flux through the *R* mode can be attributed to different isotope effects within each mode, however the values of the isotope effects estimated from the present study are 4.6 for the *R* and 6.7 for the *S* mode (Table 3.1).

Figs. 3.6a and 3.6b shows the spectra of *p*-hydroxyphenethyl alcohols isolated from incubations with rabbit plasma amine oxidase and (1*R*)- and (1*S*)-tyramines. Reactions were allowed to proceed to completion (>95%) and it can be seen for both samples that the predominant alcohol product species contain deuterium. The product obtained from incubation of (1*R*)-tyramine (Fig. 3.6a) contains 74% deuterium and that from (1*S*)-tyramine (Fig. 3.6b) 81% (Table 3.1). These reactions were also done in triplicate with 75 and 70% deuterium retained in replicate incubations with (1*R*)-tyramine and 82 and 84% for *S*-samples. Analysis as above for the bovine enzyme provides estimates of kinetic isotope effects of 2.7 and 4.6 for the *R* and *S* modes, respectively (Table 3.1). The chiral purity of the tyramine samples in this study was confirmed by incubation with porcine plasma and pea seedling amine oxidases. For both isomers, complete loss of the pro-*R* and pro-*S* hydrogens, respectively, was observed (data not shown), as with the labeled dopamines.

Temperature-dependent enantioselectivity in an alcohol dehydrogenase reaction has been recently demonstrated (23), suggesting that temperature can be a critical variable in

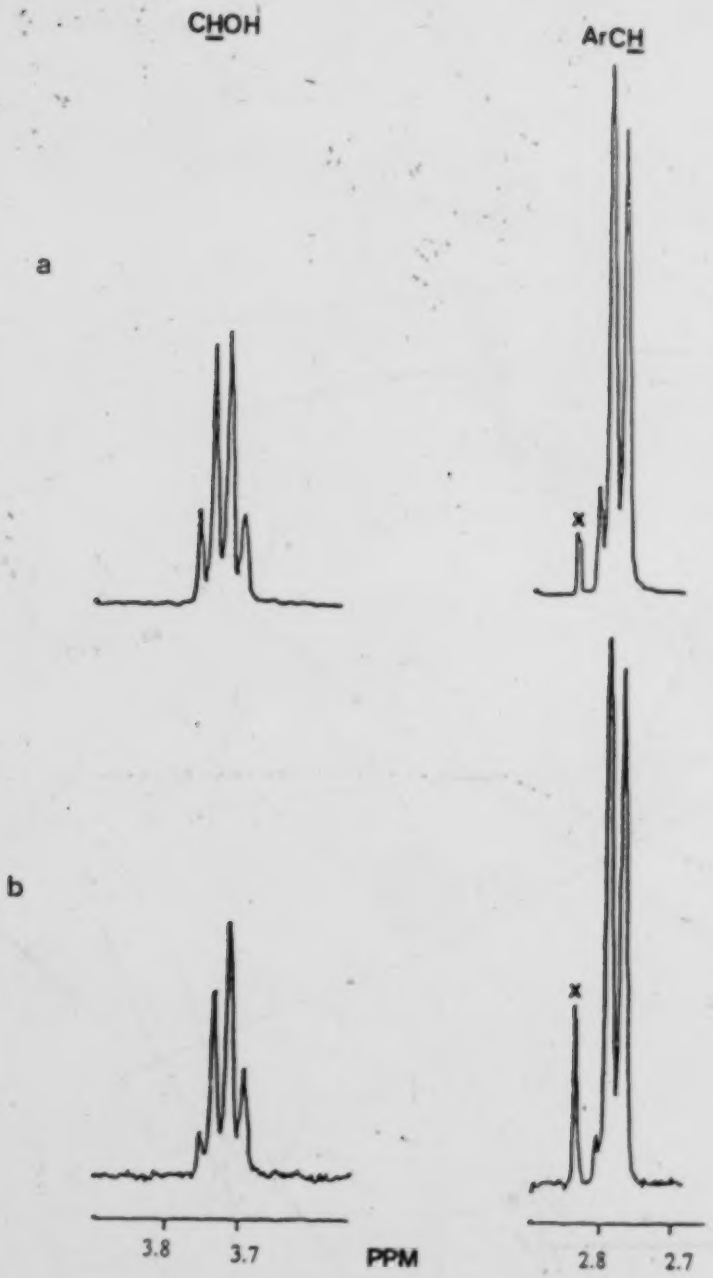
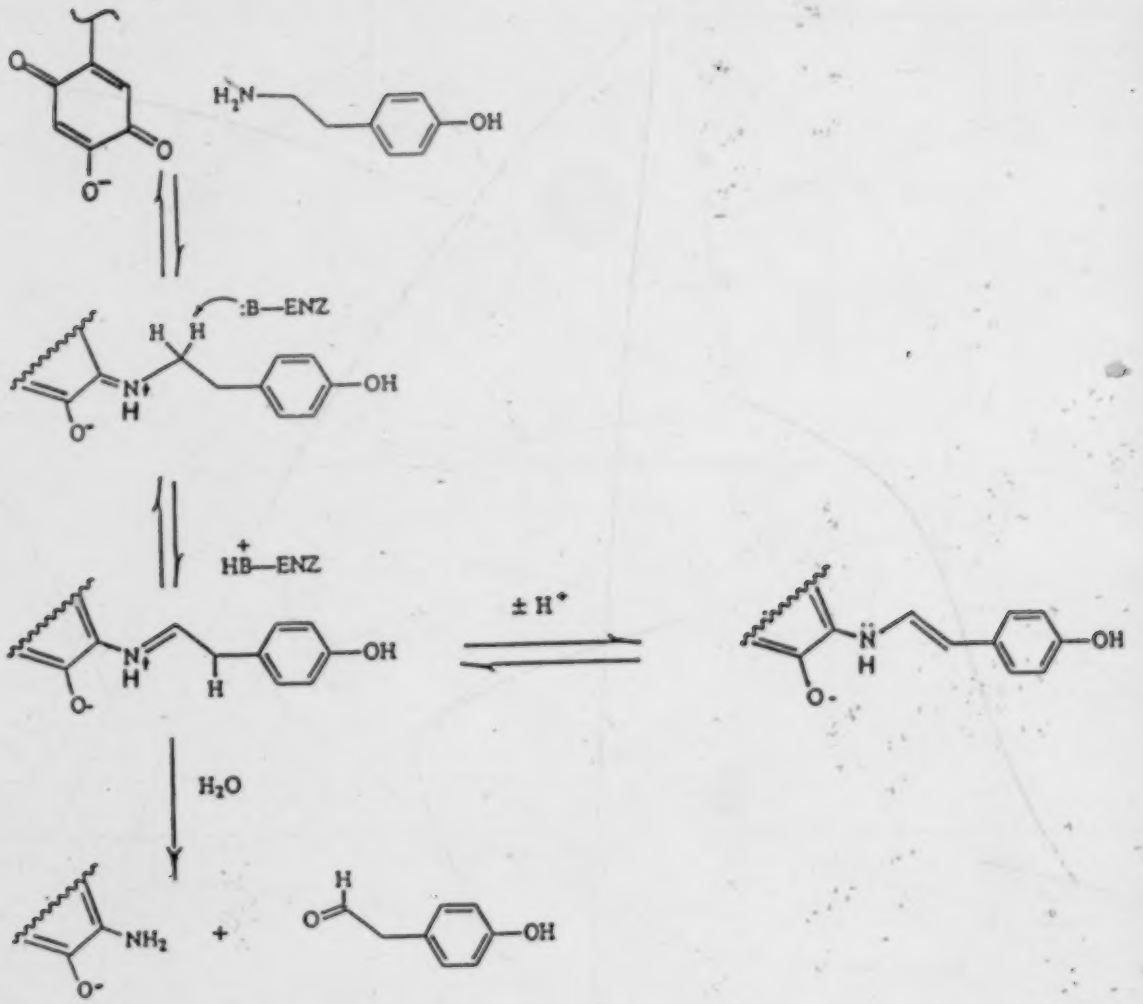


Figure 3.6. ^{1}H **NMR spectra of alcohols derived from incubations of rabbit plasma amine oxidase and stereospecifically labelled tyramines.**
(a) (1*R*)-[^{2}H]tyramine
(b) (1*S*)-[^{2}H]tyramine

asymmetric enzyme reactions. The effect of temperature on tyramine oxidation was examined by conducting a stereochemical study with bovine plasma amine oxidase at 4°C. The spectra of product *p*-hydroxyphenethyl alcohols obtained from parallel incubations with the bovine plasma enzyme are shown in Fig. 3.5. The alcohols derived from (1*R*)-tyramine were mixtures of 80% deuterated and 20% protonated species (Fig. 3.5c), while those from (1*S*)-tyramine were 93% deuterated (Fig. 3.5d). These results suggest isotope effects of 4 and 13 for the *R* and *S* modes (Table 3.1), respectively, with a small increase in the flux through the *R* mode as the temperature decreases.

The bovine and rabbit enzymes from the copper amine oxidase class both exhibit the two catalytically competent pathways for tyramine oxidation. Each of these pathways appears to have been conserved. In addition, (1*R*)-[^{2}H]tyramine exhibits a smaller kinetic isotope effect than (1*S*)-[^{2}H]tyramine in both cases. The limiting step is C_1-H bond cleavage for the pro-*S* abstraction mode in all cases, with kinetic isotope effects of 6.7 and 4.6 estimated for the enzymes. The corresponding isotope effects of 4.6 and 2.7 for the pro-*R* abstraction mode are reduced in both cases (Table 3.1). This indicates that a step other than C-H bond cleavage is partially rate limiting in the *R* mode which reduces the observed isotope effect. An aminotransferase mechanism has been proposed for the copper oxidases with initial Schiff base formation between the cofactor carbonyl and substrate amine (Scheme 3.2) (13,24). This is followed by base-catalyzed proton abstraction from C-1 of substrate such that the cofactor functions as an electron sink during catalysis, analogous to pyridoxal phosphate. Imine hydrolysis yields product aldehyde and an amino quinol as the reduced cofactor species (24).

To our knowledge, there is no precedence for all possible stereochemical reaction courses to occur within a single class of enzyme acting on the same substrate. The closest example is that of malate decarboxylase, acetolactate decarboxylase and acetoacetate decarboxylase, which have been shown to catalyze the decarboxylation of α-keto acids with net retention, inversion and racemization, respectively (25). In this instance, while the

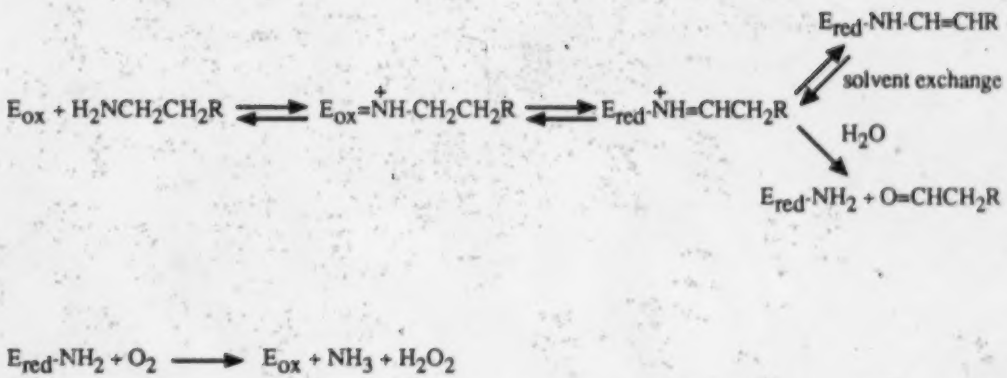


Scheme 3.2. Proposed mechanism for amine oxidase involving active site carbonyl group.

chemical transformation is the same, the substrates, acetolactate, malate and acetoacetate are different. The decarboxylases are further classified differentially by Enzyme Classification (E.C.) number.

Bovine and rabbit plasma amine oxidases clearly exhibit two different binding modes, such that a catalytic base on the enzyme can abstract a proton from C-1 of substrate with absolute and opposite stereospecificity within each mode. While it is tempting to speculate that the porcine and pea seedling enzymes have evolved such that only one of the two binding modes has been conserved in each enzyme, this cannot be confirmed in the absence of genetic and structural information on the enzymes. It is possible, however, to use additional chemical probes to establish mechanistic inter-relationships for the four enzymes. Lovenberg and Beaven (7) showed that deamination of C-2 tritiated dopamine by bovine plasma amine oxidase occurred with release of label into solvent, while reaction with the flavin-containing monoamine oxidase resulted in retention of label in the deaminated product. This was ascribed to the reversible formation of an enamine off the main enzyme reaction pathway, Scheme 3.3. Thus, solvent exchange at C-2 provides a mechanistic distinction between the copper and flavin-enzymes.

The solvent-exchange characteristics of the amine oxidase reactions catalyzed by the porcine, bovine, pea seedling and rabbit enzymes were compared by conducting the reactions using fully protonated dopamines and tyramines in ^2H_2O (Table 3.1). The ^1H NMR spectra of the alcohols obtained from these reactions are presented in Fig. 3.7. The porcine, bovine and rabbit enzymes catalyze incorporation of deuterium into C-2, but to different extents, 1.2, 0.8 and 0.7 deuteriums, respectively (Fig. 3.7a,c,e). The wash-in experiment was carried out in triplicate for the porcine enzyme and in each case 1.2 ± 0.05 atoms of deuterium were observed in the isolated products. Wash-in catalyzed by the bovine enzyme was more variable, duplicate experiments with dopamine showed 0.8 and 1.1 atoms of deuterium in product, while duplicates for tyramine both gave alcohols with 0.8 ± 0.05 atoms of deuterium. Deuterium incorporation at C-2 in the dopamine reaction



Scheme 3.3. Proposed minimal mechanism for amine oxidase reaction.

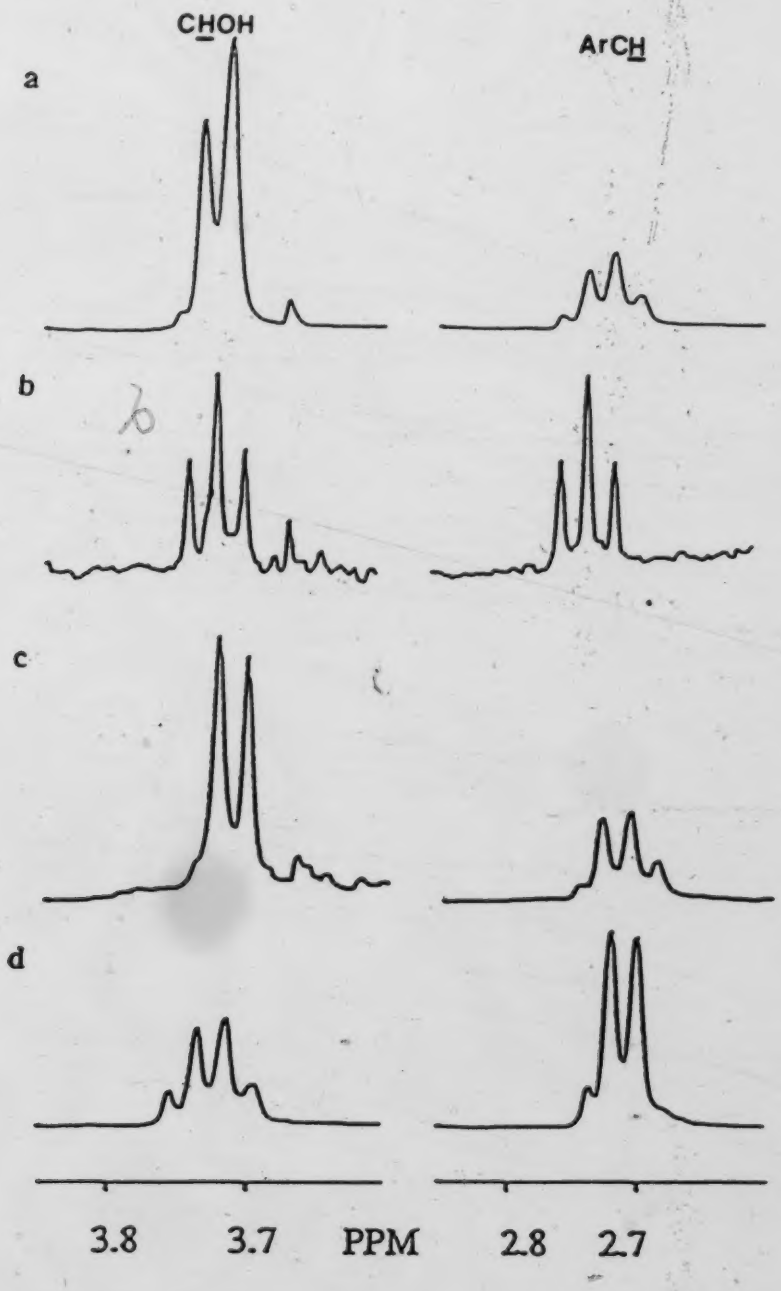


Figure 3.7. ^{1}H **NMR spectra of alcohols derived from incubations in** $^{2}H_2O$ **of**
(a) porcine amine oxidase and dopamine
(b) pea seedling oxidase and dopamine
(c) bovine amine oxidase and dopamine
(d) pea seedling amine oxidase and tyramine

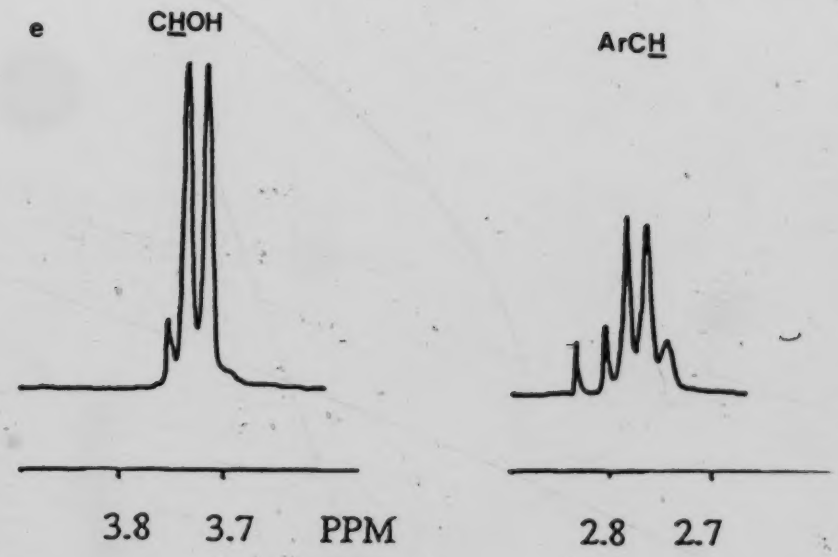


Figure 3.7. *(continued)* **^{1}H NMR spectra of alcohols derived from incubations in ^2H_2O of**
(e) rabbit amine oxidase and tyramine

catalyzed by the bovine enzyme has been shown to occur stereospecifically (13). The rabbit enzyme incorporated 0.7 ± 0.4 atoms of deuterium into tyramine (Fig. 3.7e). Our general observation of the incorporation of less than one deuterium into product would be consistent with some nonstereospecificity during wash-in, and the C-2 stereochemical studies in Chapter 4 will address the basis for this variability. No deuterium was detected in the alcohol obtained from the incubations with pea seedling amine oxidase and dopamine (Fig. 3.7b). The extent of reversibility of the wash-in step was further probed by reisolating amines from ^2H_2O incubations; in no case was deuterium found in the reisolated amines.

Incubations with unlabelled tyramine and pea seedling enzyme in ^2H_2O yielded alcohols which were 80% deuterated at C-1 (Fig. 3.7d). The degree of deuterium incorporation at C-1 was variable and ranged from 0-90%, for identical incubations with different enzyme preparations. This wash-in is attributed to solvent exchange into either the NADH or the cyclohexanol used in the coupling reaction with one batch of pea seedling enzyme which was used only in the solvent exchange experiment. The reasons for such variation in pea seedling preparations are unclear at present, however in no instance was there any detectable deuterium incoporated at C-2.

From the ^2H_2O experiments, clear distinctions can be seen to exist between the four copper enzymes, with respect to substrate specificity, position and extent of deuterium incorporation into products. The differential incorporation of label into 3,4-dihydroxyphenethyl alcohols and tyrosols seen for bovine, porcine and rabbit amine oxidases arises from different ratios in the relative rates of hydrolysis and enamine formation. Although the pea seedling enzyme resembles the flavin-containing monamine oxidases which lack the enamine exchange pathway the spectral properties and presence of TOPA (Klinman, J.P., unpublished results) preclude flavin as the cofactor in this enzyme.

A collaborative study towards the structural analysis of the cofactor is in progress to reconfirm the presence of TOPA in the porcine and pea seedling amine oxidases. As a

result of the wash-in ability of the plasma enzymes, the stereochemical relationship between proton abstraction at C-1 and protonation at C-2 for the porcine enzyme by configurational analysis was studied. Previous studies had established that both processes were catalyzed by a single base in bovine plasma amine oxidase (13). These studies are dealt with in the next chapter.

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4. The Stereochemistry of Plasma Amine Oxidase Reaction at C-2 Position of Tyramine

Introduction

Copper-containing plasma amine oxidases catalyze the oxidative deamination of primary amines, converting them to their corresponding aldehydes and releasing ammonia and hydrogen peroxide (Equation 4.1):

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2 \qquad [4.1]$$

In addition to copper, an organic cofactor is also reported to be present in these enzymes (2,3). Recently, 6-hydroxydopa or topa (Chapter 1, Fig. 1.2) has been identified as the cofactor in bovine plasma amine oxidase (4). Unpublished results from the same lab have suggested that the same organic moiety is present in porcine plasma amine oxidase.

So far, these plasma enzymes have exhibited unusual stereochemistries in their reactions with substrates (5). The porcine plasma enzyme abstracts the pro-*R* hydrogen at C-1 of tyramine and dopamine (6) while the bovine enzyme, the first of its kind to show nonstereospecificity in oxidative deamination of tyramine and dopamine, portrayed a rare example of mirror image binding and catalysis (6,7,9). However, this same bovine enzyme is pro-*S* specific for abstraction of hydrogen at C-1 of benzylamine (8). Recent reports on the enzymes isolated from sheep and rabbit plasma have added two more examples of nonstereospecificity of oxidation of the C-1 protons of tyramine (5) by plasma amine oxidases.

Generally, there is a conservation of substrate binding geometry in the active site of related enzymes. This conservation is dictated by both evolutionary pressures and the underlying chemistry of the transformations, and this results in the conservation of substrate orientation relative to the catalytic bases such that there is stereochemical uniformity for any class of enzymes. This kind of uniformity is lacking in copper-containing amine oxidases, in their oxidation of C-1 protons of tyramine and dopamine. However, a distinct and consistent pattern exists in the solvent exchange properties of these

Table 4.1. Summary of stereochemical studies on copper amine oxidases.

Enzyme Source	*C-1 Proton Abstraction*	*C-2 Solvent Exchange*	*Reference*
Porcine plasma	pro-*R*	Yes	(6)
Bovine plasma	nonstereospecific	Yes	(6,7,9)
Sheep plasma	nonstereospecific	Yes	(5)
Rabbit plasma	nonstereospecific	Yes	(5)
Pea seedling	pro-*S*	No	(6,13)
Soybean seedling	pro-*S*	No	(5)
Chick pea seedling	pro-*S*	No	(5)
Pig kidney	pro-*S*	No	(5)

same enzymes (5,6). A summary of the stereochemical studies of some of these copper amine oxidases is shown in Table 4.1.

The solvent exchange characteristics of these copper amine oxidases rely on the enzyme's ability to go through the imine-enamine tautomerization pathway which lies off the main catalytic pathway of the enzyme reaction (Scheme 4.2). The rate of hydrolysis of the imine relative to the enamine formation dictates whether or not solvent will exchange into the product. So far, the plasma amine oxidases from bovine, porcine, sheep and rabbit have shown this distinct solvent exchange property, although the extent of solvent incorporation differs for each enzyme.

The bovine plasma amine oxidase is the most studied enzyme. Solvent exchange into C-2 of dopamine in the reaction involving the bovine enzyme was reported to be pro-*R* specific (7,9). However, Farnum and Klinman (9) reported that the loss of label from C-2 of dopamine in a similar reaction involving the same bovine enzyme proceeded nonstereospecifically. They concluded that enamine protonation was not the microscopic reverse of the imine exchange, an observation which appeared to violate the principle of microscopic reversibility. A comparison of the relationship between the C-H bond cleavages at C-1 and C-2 of dopamine resulted in the observation that a single basic residue at the active site of the bovine enzyme catalyzed the cleavages in a syn manner.

In this chapter the stereochemistry of hydrogen activation at C-2 of tyramine by porcine and bovine plasma amine oxidases is investigated.

Materials

Tyramine•HCl was purchased from Eastman Kodak Co., (2*R*)-[^{2}H]tyramine and ascorbic acid were gifts from Ms. C. Scaman and Dr. Linus Pauling. 2-(4-hydroxyphenyl)ethyl-2,2-$^2H_2$-amine•HCl was obtained from Merck Inc. Dimethylaminopyridine (DMAP), benzyl chloride, toluene sulfonyl chloride (TsCl) which was recrystallized, fumaric acid ($2Na^+$), catalase, dopamine β-hydroxylase (DBH) and Dowex 50(H^+)-X8

were purchased from Sigma Chem. Co. Hydrazine hydrate (99-100%), palladium on charcoal (5%), and silica gel 60 (230-400 mesh) were obtained from BDH Chem. Ltd. N,N-dimethylformamide (DMF) from Caledon Laboratories was distilled, then stored over molecular sieves and protected from light. Pyridine and aluminum sheet silica gel 60 F_{254} TLC plates were purchased from Terochem Laboratories. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) and acetonitrile-$^2H_3$ (99%) were obtained from Aldrich Chem. Co. Potassium phthalamide, acetone-$^2H_6$ (99.9%), methanol-$^2H_4$ (99.8%) and chloroform-^2H (99.8%) were purchased from General Intermediates of Canada. All other chemicals and solvents (freshly distilled before use) were reagent grade and purchased form Aldrich, Sigma or Fisher Scientific. The distilled deionised water used throughout the experiments was from the Milli-Q system.

Methods

A Hewlett-Packard Diode Array Spectrophotometer was used for all spectrophotometric measurements. A Beckman gradient liquid chromatographic system, attached to a Bio-Rad UV detector and fitted with a Waters C-18 radial pak cartridge (column) was used for HPLC analysis. A Bruker WM-360 ^1H NMR instrument was used to record all NMR spectra at 360 MHz, courtesy of Dr. O. Hindsgaul, S. Crawley and L. Newton. The spectra were accumulated into 16 K of computer memory using a 45° pulse with 7 s delays between pulses. Longer relaxation delays did not affect the relative intensities of the signals. The intensity and multiplicity of these signals are described using abbreviations: s = singlet; d = doublet; t = triplet; m = multiplet, and the chemical shifts of the signals relative to the residual signals from the NMR solvents are also described in ppm.

Enzymes

Fresh porcine blood was purchased from Gainers Ltd. (Edmonton, AB), and the amine oxidase was isolated as described fully in Chapter 2. The specific activity of the porcine enzyme was 0.059 unit/mg. Bovine plasma amine oxidase was purchased from

Sigma Chem. Co. (St. Louis, MO) and used without further purification, and its specific activity was 0.067 unit/mg.

Enzymatic synthesis of C-2 [^{2}H] 4-hydroxyphenethyl alcohol (tyrosol); solvent incorporation experiments

Incubations were carried out as described by Battersby *et al.* (8), with minor modifications. About 1.0 unit each of porcine or bovine plasma amine oxidases (previously exchanged in ^2H_2O buffer) was incubated with 130 µmole (22.6 mg) of tyramine hydrochloride and reacted at 25°C with 260 µmole (207 mg) of NADH in a total of 10 ml of 100 mM deuterated sodium or potassium phosphate buffer, pD 6.9. The incubation mixture also contained 27,900 units of catalase and 30 units (15 mg) of equine liver alcohol dehydrogenase. The reaction was allowed to go to 100% completion, by monitoring the decrease in absorbance of NADH at 340 nm.

The product, tyrosol, diluted to 2 x its volume with distilled water, was extracted from the reaction mixture with an equal volume of ethyl acetate in a separatory funnel. The procedure was repeated to ensure maximum extraction of tyrosol into the ethyl acetate. The organic fraction was evaporated to dryness on a rotary evaporator, and all remaining NADH and accumulated NAD were removed by flash chromatography on a column of 2 g of silica gel, using ethyl acetate:*n*-hexane (2:1) as solvent. A sample of the eluate was spotted for TLC on silica plates, and run in the same solvent to establish the absence of free amine. An R_f of 0.53 was obtained for the alcohol. The residue, after removal of the solvent and freeze drying, was analysed by ^{1}H NMR at 360 MHz in a 4:1 $(^2H_3C)_2CO$:^2H_2O (deuterated acetone:deuterium oxide) solvent (Fig. 4.1a,b).

Similar incubation mixtures were set up in sodium or potassium phosphate buffer, pH 7.0, this time containing C-2 dideuterated tyramine•HCl and protonated bovine plasma amine oxidase. Product was extracted the same way and showed a similar R_f value. Fig. 4.1c shows the NMR spectrum.

Chemical conversion of C-2 [^{2}H]4-hydroxyphenethyl alcohol (tyrosol) **1** to C-2 [^{2}H]4-hydroxyphenethyl amine (tyramine)

i. Protection of phenolic groups

The method by Farnum and Klinman (9) with modifications by Dr. O. Hindsgaul[1] was used in the chemical synthesis of amines from their corresponding alcohols (Scheme 4.1). About 10-15 mg each of C-2 [^{2}H]tyrosol residues from the porcine and bovine incubations from the preceeding step was dissolved in 1 ml distilled methanol in a screw cap vial. About 2-3 equivalents each of DBU (23.2-34.8 µl) and benzyl chloride (18-27 µl) were injected in succession into the vial, which was then capped tightly. The reaction mixture was incubated overnight at 65°C in a stirred oil bath. Excess reagents (2-3 equivalents of DBU and benzyl chloride) were added after 6-7 h of incubation. At the end of the reaction, 1 ml 1 N NH_4OH was added to the cooled reaction mixture, which was stirred for 30 min and mixed with 30 ml dichloromethane (CH_2Cl_2) before pipetting into a separatory funnel. The mixture was washed in succession with 30 ml each of H_2O, 1 N HCl and more H_2O. The CH_2Cl_2 phase was filtered through Whatman No. 1 paper (wetted with CH_2Cl_2) and the organic phase removed on a rotary evaporator. The residue, dissolved in 1 ml ethyl acetate:*n*-hexane (1:1) and the flask rinsed 3 x with the same volume of solvent, was chromatographed on a short silica gel 60 column. The course of reaction was followed by TLC in ethyl acetate:*n*-hexane:ethanol (10:10:1) solvent on a silica plate. The R_f of the purified benzylated tyrosol was 0.7. The ^{1}H NMR signals in $CDCl_3$ (δ in ppm) are as follows: 2.8 (t, 1H); 3.8 (d, 2H); 5.0 (s, 2H); 6.9 (m, 2H); 7.1 (m, 2H); 7.24 (s, $CDCl_3$); 7.4 (m, 5H).

ii. Conversion of benzyloxyphenethyl alcohol **2** *to the tosyl ester* **3**

The benzyloxyphenethyl alcohol residue (64-108 µmole) was dissolved in 500 µl of CH_2Cl_2, then 4 equivalents of DMAP (256-432 µmole) and 3 equivalents of toluene

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1 Dr. O. Hindsgaul is a collaborator in the Chemistry Department of the University of Alberta.

sulphonyl chloride (192-324 μmole), each dissolved in a minimum amount of CH_2Cl_2 (about 250 μl), were added. The mixture was stirred at room temperature. After 1 h, TLC of the mixture (in EtOAc:Hexane:EtOH, 10:10:1) showed incomplete conversion, therefore excess reagents in the same amounts were added. At the end of the reaction (2.5-3 h), 500 μl of pyridine:H_2O (4:1) was added to the reaction mixture and stirred for 30 min at room temperature. The tosylated product was isolated by extraction into 30 ml CH_2Cl_2 in a separatory funnel, then washed sequentially with H_2O, 1 N HCl and $NaHCO_3$ (30 ml). The organic fraction was filtered through wetted Whatman paper and evaporated to dryness on the rotary evaporator. TLC of the residue, run in EtOAc:Hexane:EtOH (10:10:1), showed an R_f of 0.87. The ^1H NMR signals in $CDCl_3$ (δ in ppm) are as follows: 2.4 (s, 3H); 2.9 (t, 1H); 4.2 (d, 1H); 5.0 (s, 2H); 6.8 (m, 2H); 7.0 (m, 2H); 7.24 (s, $CDCl_3$); 7.4 (m, 5H); 7.6 (d, 4H).

iii. Conversion of tosyl ester **3** *to the phthalamide* **4**

DMF (0.6 ml) was added to the residue from the preceeding step into a screw cap vial. Potassium phthalamide (4 equivalents, 40-60 mg) was added, and the mixture stirred overnight at 60°C. The product from the reaction mixture was extracted into CH_2Cl_2 (30 ml) after the mixture had cooled to room temperature, and then washed sequentially with 30 ml H_2O with ice (2 x), 30 ml 1 N NaOH with ice (2 x), and 30 ml H_2O (2 x). The CH_2Cl_2 fraction was filtered and evaporated to dryness. TLC of the phthalamido product in 2% MeOH in CH_2Cl_2 showed an R_f of 0.79. The ^1H NMR signals in $CDCl_3$ (δ in ppm) are: 2.9 (t, 1H); 3.9 (d, 2H); 5.0 (s, 2H); 6.9 (m, 2H); 7.1 (m, 2H); 7.24 (s, $CDCl_3$); 7.4 (m, 5H); 7.7 (m, 2H); 7.8 (m, 2H).

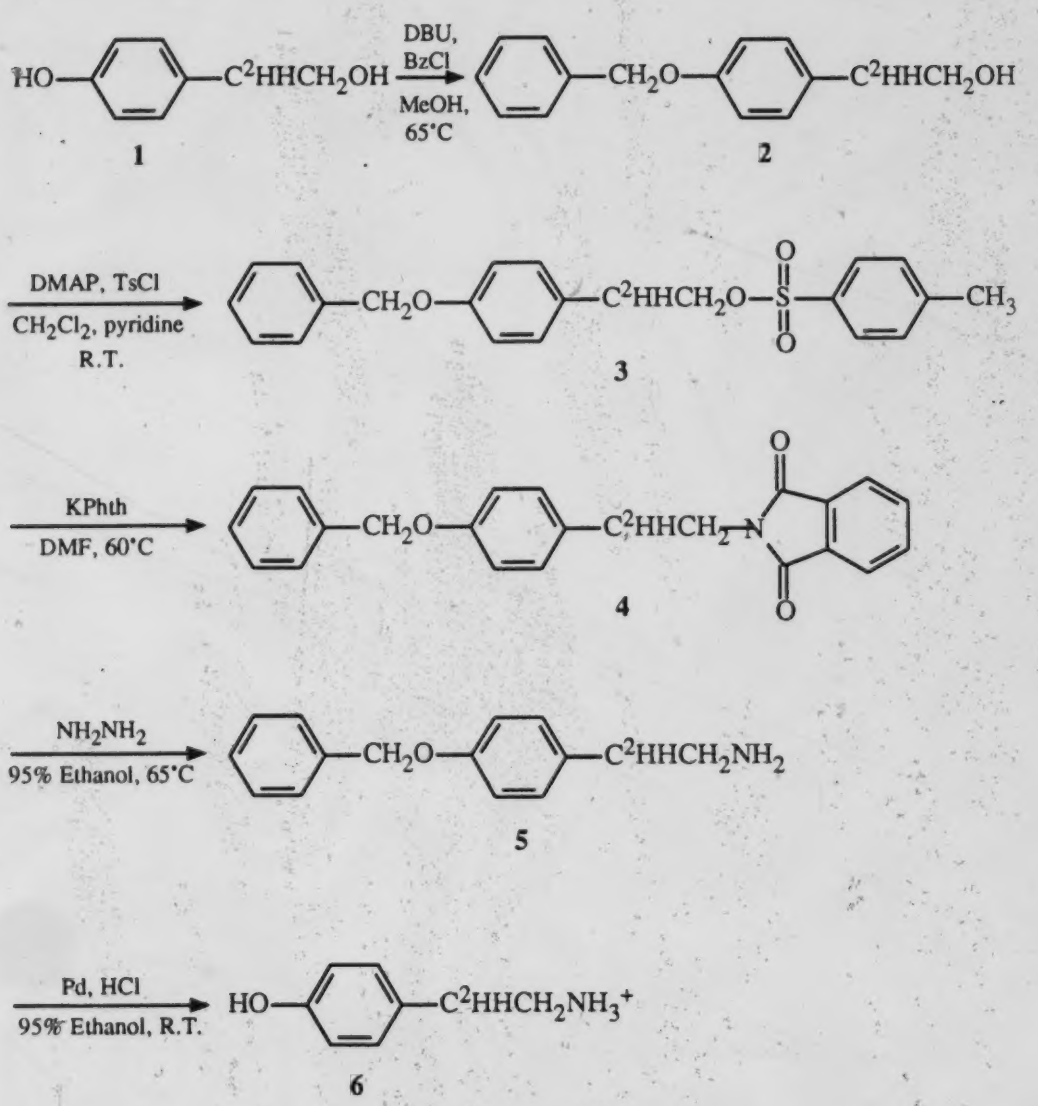
iv. Conversion of phthalamide product **4** *to benzylated amine* **5**

Ethanol (95%, 1 ml) was pipetted into the screw cap vial containing the phthalamido product. Hydrazine hydrate (100 μl) was added and the mixture was stirred overnight at 65°C. Benzylated tyramine was isolated from the cooled reaction mixture after

the ethanol had been evaporated, the residue dissolved in CH_2Cl_2 (30 ml), and the CH_2Cl_2 fraction washed sequentially with 1 N NaOH (30 ml, 2 x), H_2O (30 ml, 2 x), then filtered through Whatman paper and evaporated to dryness. TLC on silica plate of the benzylated amine in butanol:acetic acid:H_2O (12:3:5) solvent showed an R_f of 0.7. The silica plate was sprayed with ninhydrin to confirm the amine spot. The ^1H NMR signals in CD_3OD (δ in ppm) are: 2.7 (t, 1H); 2.9 (d, 1H); 3.3 (CD_3OD); 5.0 (s, 2H); 6.8 (m, 2H); 7.0 (m, 2H); 7.2 (m, 5H).

v. Hydrogenation of benzylated amine **5** *to tyramine* **6**

The residue of the benzylated amine was dissolved in 95% ethanol (2 ml). About 10-15 mg of 5% palladium on charcoal (10-15 mg), made into a slurry with 95% ethanol (1 ml), was added to the benzylated amine solution, followed by 3 drops of 1 N HCl. The mixture was stirred under H_2 gas overnight (2 psi). At the end of the reaction, the mixture was filtered, the palladium residue rinsed several times with 1 ml portions of MeOH, and the filtrate evaporated to dryness. The amine residue was redissolved in H_2O, loaded onto about 2 ml Dowex 50(H)-X8, previously washed in bulk (40g) with 2 l of 2 M NaOH, H_2O, 6 M HCl and finally H_2O until the eluate was neutral to pH paper. The loaded column was rinsed with 100 ml H_2O and the tyramine eluted with 3.5% NH_4OH solution and evaporated to dryness. TLC of C-2 [^2H]tyramine was done in butanol:acetic acid:H_2O (12:3:5) solvent and the R_f was 0.63. The ^1H NMR spectra of the amines are shown in Fig. 4.2. The overall yield from the beginning of the synthesis ranged from 20-33%. The chemical synthesis of the enzymatically derived C-2 [^2H]tyrosols to C-2 [^2H]tyramines is shown in Scheme 4.1.



Scheme 4.1. Chemical conversion of C-2 [^{2}H]tyrosol to C-2 [^{2}H]tyramine

Enzymatic conversion of C-2 [^{2}H]tyramine into octopamine with dopamine β-hydroxylase

About 1-2 mg each of C-2 [^{2}H]tyramines, synthesized from the enzymatically-derived C-2 [^{2}H]tyrosols, were incubated at 37°C for 48 h in 2.5-5.0 ml of 50 mM NaOAc buffer, pH 5.0, containing ascorbic acid (10 mM), fumaric acid ($2Na^+$ salt, 10 mM) and 0.26 unit of dopamine β-hydroxylase. Octopamine production was assayed spectrophotometrically at 330 nm (10), and the reaction was allowed to go to 100% completion. The reaction mixture was mixed with 0.5-1.0 ml of 3 M TCA before loading it onto a previously equilibrated Dowex 50(H)-X8 column. The column was washed with 100 ml water and octopamine eluted with 3.5% NH_4OH. The eluate was evaporated to dryness, dissolved in 5% MeOH in 1% acetic acid, then purified on the HPLC, using the same solvent as the mobile phase at a flow rate of 1 ml/min at room temperature. Octopamine eluted with a retention time of 3.2 min. The purified octopamine was evaporated to dryness on the rotary evaporator, redissolved in deuterated acetonitrile (C^2H_3CN) and evaporated to dryness (2 x). The residue was finally dissolved in C^2H_3CN:^2H_2O (4:1) for NMR analysis.

Oxidation of (2*R*)-[^{2}H] and (2*S*)-[^{2}H]tyramines by porcine and bovine plasma amine oxidases (wash-out experiments)

Incubations of (2*R*)-[^{2}H] and (2*S*)-[^{2}H]tyramine (0.4-0.5 mg) with porcine and bovine plasma amine oxidases (0.2 unit) were set up separately in a total volume of 1 ml in 100 mM phosphate buffer, pH 7.0, a modification of the method by Battersby *et al.* (8). The reaction mixtures also contained catalase (12,090 units), liver alcohol dehydrogenase (3.2 units) and NADH (8.5 mg). The production of tyrosol was followed spectrophotometrically by the reduction of NADH at 340 nm. At 100% completion of reaction, the alcohol was isolated by extraction into ethyl acetate and chromatographed on silica (2 g) with ethyl acetate:hexane (2:1) as solvent. The eluate was dried on the rotary evaporator, lyophilized, and then dissolved in $(^2H_3C)_2CO$:^2H_2O (4:1) for NMR analysis.

Steady state kinetics for tyramine oxidation with porcine plasma amine oxidase

The reaction was carried out in a coupled enzyme system at 25°C with both diprotonated and C-2 dideuterated tyramine at concentrations ranging between 0.57 to 22.9 mM, in a total volume of 0.915 ml. Incubation mixtures also contained 0.14 unit liver alcohol dehydrogenase, 818.4 units catalase, 0.31 μmole NADH and 0.012 unit porcine amine oxidase. The rate of oxidation was measured spectrophotometrically by the decrease in absorbance of NADH at 340 nm (extinction coefficient of NADH being 6.23×10^3 M^{-1} cm^{-1}). The initial rate data obtained were fitted to the expression $v = V_{max}[S]/(K_m + [S])$, based on the FORTRAN program of Cleland (11).

Results and Discussion

Solvent incorporation at C-2 of tyramine

Previous studies (6) of the reaction of tyramine in ^2H_2O with porcine and bovine plasma amine oxidases in a coupled reaction with NADH and alcohol dehydrogenase, showed the incorporation of deuterium at C-2 of the tyrosol formed. The deuterium content of the product tyrosol was estimated from ^1H NMR spectra. For the porcine enzyme, experiments were done in triplicate and an average value of 1.2 ± 0.05 atoms of deuterium were incorporated from solvent. The results in the present experiment duplicated the deuterium content observed in the previous study (6). Fig. 4.1a shows the ^1H NMR spectrum of the tyrosol. The signal at C-2 (near 2.7 ppm) indicates 2 overlapping triplets, while C-1 (near 3.7 ppm) shows a doublet superimposed on a triplet. The major product, which is C-2 monodeuterated tyrosol, is distinguishable from the diprotonated species by an upfield shift of 0.02 ppm, due to the C-2 deuterium isotope effect. This upfield shift had previously been seen in tyrosols produced from the oxidative deamination of both (1*R*)- and (1S)-[^2H]tyramines (6). Upfield shifts due to heavy isotope substitution or replacement of a proton in a C-H bond are known to cause such shifts, which in this case show a β-deuterium isotope effect (12). Similar signals are seen in Fig. 4.1b, which is the alcohol product from the wash-in experiment involving tyramine and bovine plasma amine oxidase. A consistent estimated deuterium content of 1.1 atoms was obtained, which is in the range of values previously estimated for the bovine enzyme's reaction with tyramine and dopamine (6). The estimated deuterium contents in duplicate reactions of bovine enzyme with dopamine in the previous experiment were 0.8 and 1.1 atoms, but the reaction with tyramine gave values of 0.8 ± 0.05 atoms (6). Although the variable values could be due to slight errors in the estimation of the deuterium, they may also indicate some small nonstereospecificity in the deuterium washed-in at C-2 of the substrate by the bovine enzyme, and this could be proven in the stereospecific pro-*R* reaction using DBH. Previous

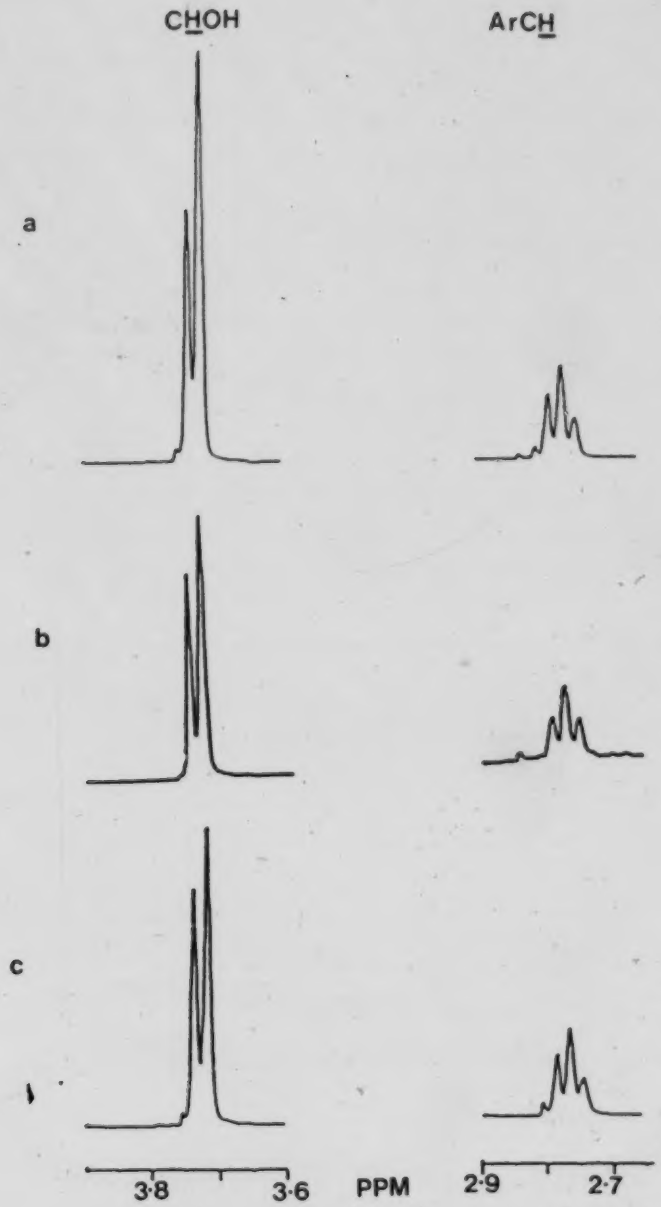


Figure 4.1. ^{1}H NMR spectra of C-2 [^{2}H]tyrosol: solvent incorporation

(a) Porcine amine oxidase / $^{2}H_2O$
(b) Bovine amine oxidase / $^{2}H_2O$
(c) Bovine amine oxidase / H_2O

reports, however, have shown a pro-*R* stereospecific incorporation of label by the bovine enzyme (7,9).

Fig. 4.1c shows the signals observed for the wash-in reaction using the bovine enzyme and the dideuterated tyramine in H_2O. The NMR spectrum is similar to Fig. 4.1b, except that the reaction for Fig. 4.1b involved diprotonated tyramine and ^2H_2O. The signals in Fig. 4.1c indicate that deuterium in one of the C-2 positions was cleaved off by the enzyme. The signal for C-2 near 2.7 ppm, as was observed in Fig. 4.1a and b, is made up of two overlapping triplets, while the C-1 signal near 3.7 ppm shows a doublet superimposed on a triplet. The major product, as in the other two cases (Fig. 4.1a and b), is the C-2 monodeuterated alcohol species, which is shifted about 0.02 ppm upfield from the minor diprotonated alcohol species. Again, the deuterium content was estimated in this experiment (with C-2 dideuterated tyramine in H_2O) to be 1.1 atoms, which is the same as found in Fig. 4.1b. A summary of the deuterium incorporated into tyramine by porcine and bovine plasma amine oxidases is shown in Table 4.2.

The different extents of deuterium incorported by the porcine and bovine enzymes into tyrosol (reaction in ^2H_2O), and the amount retained by the bovine enzyme in tyrosol (reaction in H_2O) may be due to relative rates of hydrolysis and enamine formation (Scheme 4.2). Solvent exchange in this scheme is seen to occur off the main enzyme pathway, where the imine-enamine tautomerization dictates the exchange. The slower the rate of imine hydrolysis relative to enamine formation, the greater the amount of solvent incorporated into the C-2 position. However, failure of incorporation of solvent into C-2 position, as was the case for pea seedling amine oxidase (6), implied that the enamine pathway was bypassed in the enzyme reaction.

Table 4.2. Characteristics of enzymatic products from wash-in and DBH reactions[1], and from wash-out reactions[2] as analyzed by ^1H NMR*.

[1]*Enzyme / Stereochemistry*	*Substrate / Product*	*Deuterium content at C-2* # *atoms*	%	
Porcine	[a] C-2 [^2H]tyrosol	1.20±0.05	100	
Nonstereospecific	[b] C-2 [^2H]tyramine	1.20±0.05	100	
	[c] Octopamine	0.5	42	(58 loss)
Bovine	[a] C-2 [^2H]tyrosol	1.10	100	
Pro-*R*	[b] C-2 [^2H]tyramine	1.10	100	
	[c] Octopamine	0	—	(>96 loss)
	[d] Octopamine	1.10	>97	

[2]*Enzyme / Stereochemistry*	*Product*	*Deuterium content at C-2* *% retained*	*% lost*	*KIE*
Porcine	(2*R*)-tyrosol	[e] 79	21	3.8
Nonstereospecific		[f] 79	21	3.8
	(2*S*)-tyrosol	62	38	1.6
Bovine	(2*R*)-tyrosol	73	27	2.7
Nonstereospecific				
	(2*S*)-tyrosol	66	34	1.9

* experiments done in at least duplicates

a Tyrosol from wash-in reaction.

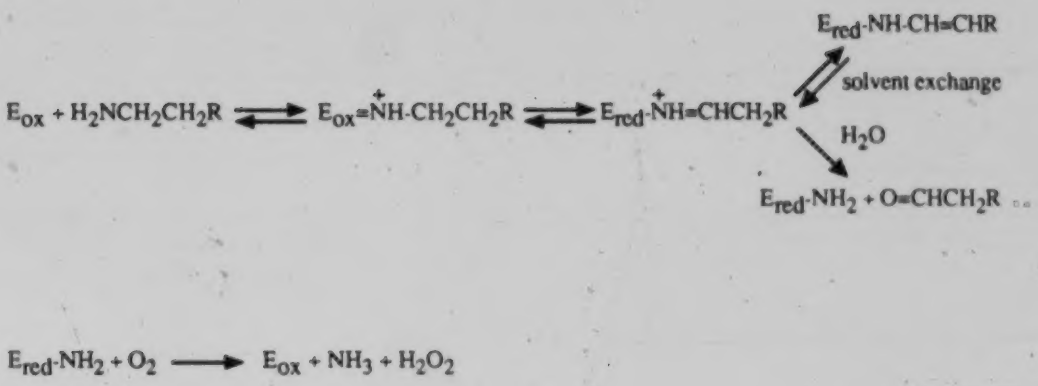
b Tyramine chemically synthesized from washed-in tyrosol.

c Octopamine from DBH reaction with tyramine from b (diprotonated tyramine as substrate for wash-in reaction).

d Octopamine from DBH reaction (dideuterated tyramine as substrate for wash-in reaction).

e (2*R*)-tyramine substrate produced after the chemical synthesis of enzymatically derived (2*R*)-tyrosol from an incubation of semicarbazide sensitive amine oxidase from porcine aorta in ^2H_2O.

f Same as above, but from bovine plasma amine oxidase in ^2H_2O.



Scheme 4.2. Proposed minimal mechanism for amine oxidase reaction.

Hydroxylation of C-2 [^{2}H]tyramine by dopamine β hydroxylase (DBH): Stereochemistry at the C-2 position

In order to determine the stereochemistry of the C-2 [^{2}H]tyrosols from both porcine and bovine plasma amine oxidases, these alcohols were converted to their corresponding tyramines by the chemical synthesis indicated under the methodology section. Fig. 4.2 shows the ^{1}H NMR spectra of the final amines. Estimation of the deuterium content in the tyramines, as was done for the C-2 [^{2}H]alcohols, gave values of 1.2 (porcine enzyme) and 1.1 (bovine enzyme) atoms, which implied that there was no loss of deuterium at the C-2 position during the chemical conversion of the alcohols to the amines. Studies involving the hydroxylation of dopamine by dopamine β hydroxylase (DBH) were reported by Battersby *et al.* (13) in their elucidation of the stereochemical course of this enzyme's reaction. They observed that DBH was pro-*R* specific when hydroxylating at the C-2 position of dopamine. Hydroxylation of substrate by DBH occurs with retention of configuration (Scheme 4.3). The stereospecificity of this reaction was made use of when the C-2 [^{2}H]tyramines shown in Fig. 4.2 were converted to octopamines by DBH, a reaction where an -OH group was incorporated into the 2*R* position of the tyramines. The NMR spectra of the octopamine products are shown in Fig. 4.3.

Octopamine derived from tyramine (from porcine enzyme syntheses; Fig 4.3a) shows two sets of doublets centred around 4.6 ppm, and a complex group of signals (multiplet) around 2.8 ppm. These signals are the result of two species of octopamine present in the product, one protonated at C-2 and the other deuterated at C-2. The signal near 4.6 ppm is due to the proton attached to C-2 of the protonated octopamine. This proton is in the pro-*S* position since DBH only removed the pro-*R* hydrogen at the C-2 position and replaced it with -OH. The presence of the -OH group (at the chiral C-2 centre), adjacent to the $-NH_2$ group at C-1, creates a nonequivalent magnetic environment for the C-1 protons. This makes the C-1 protons magnetically inequivalent. Therefore, the

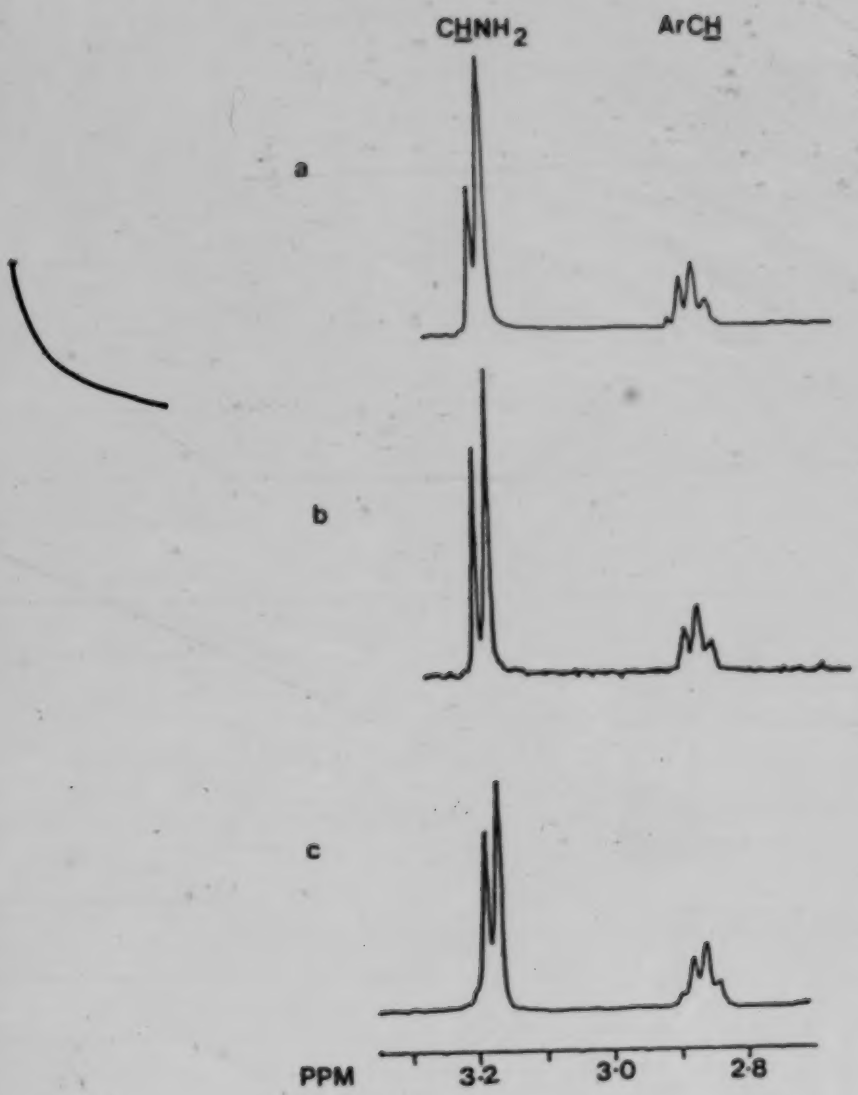
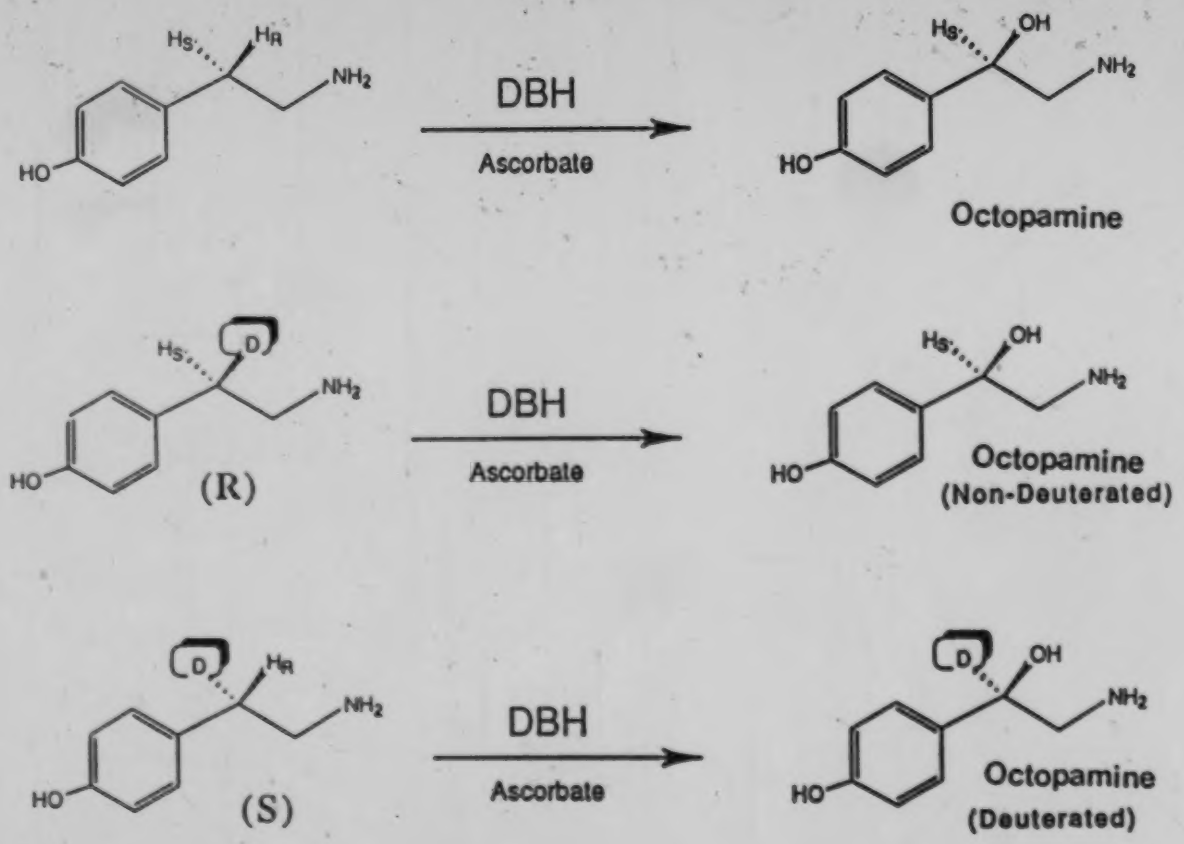


Figure 4.2. ^{1}H NMR spectra of C-2 [^{2}H]tyramine chemically synthesized from enzymatically-derived C-2 [^{2}H]tyrosol.
(a) Porcine amine oxidase / $^{2}H_2O$
(b) Bovine amine oxidase / $^{2}H_2O$
(c) Bovine amine oxidase / H_2O



Scheme 4.3. Use of dopamine β-hydroxylase to establish stereochemistry of deuteration at C-2.

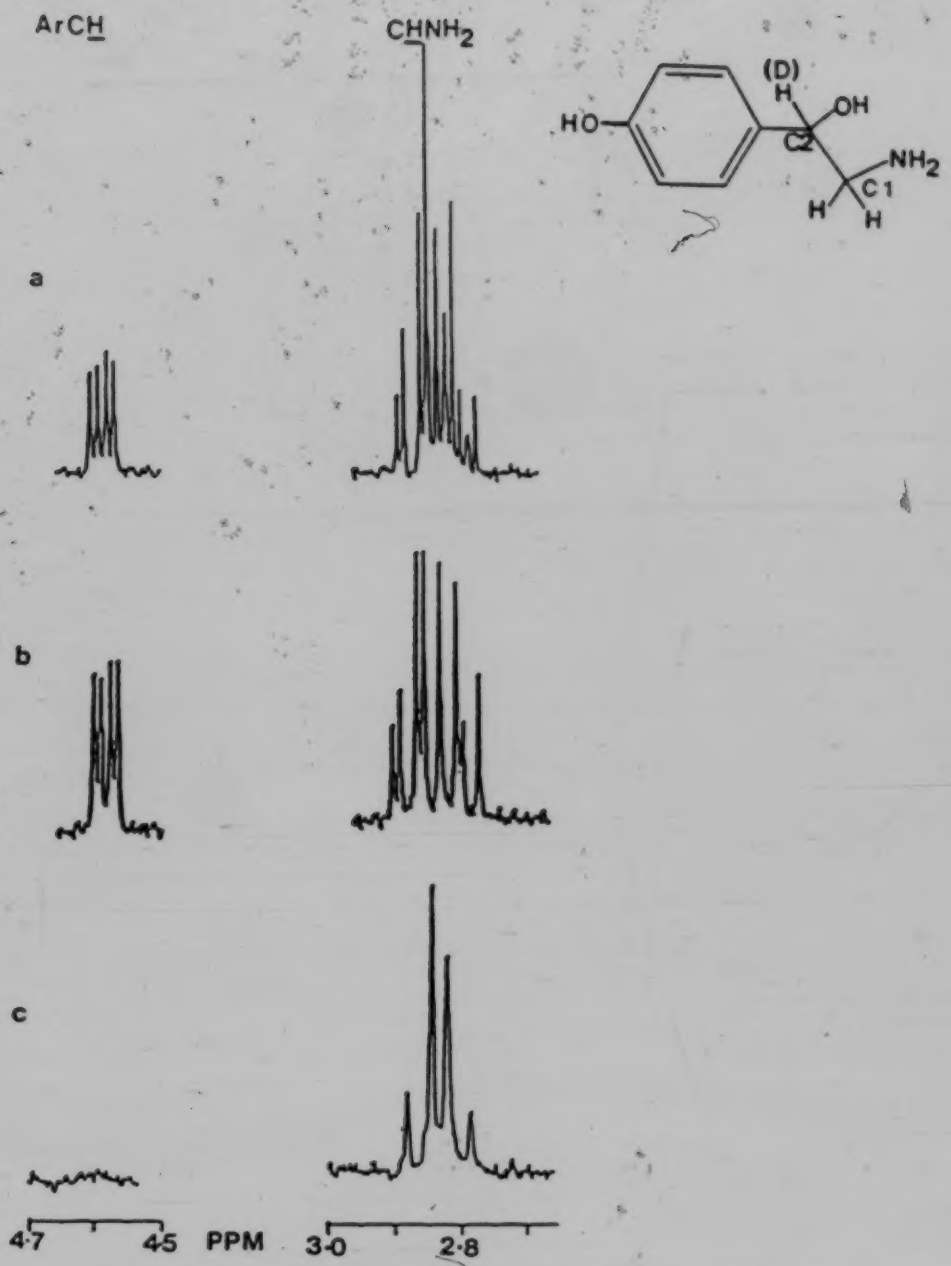


Figure 4.3. ^{1}H NMR spectra of octopamine from DBH reaction with C-2 [^{2}H]tyramine chemically derived from C-2 [^{2}H]tyrosol.

(a) Porcine amine oxidase / $^{2}H_2O$
(b) Bovine amine oxidase / $^{2}H_2O$
(c) Bovine amine oxidase / H_2O

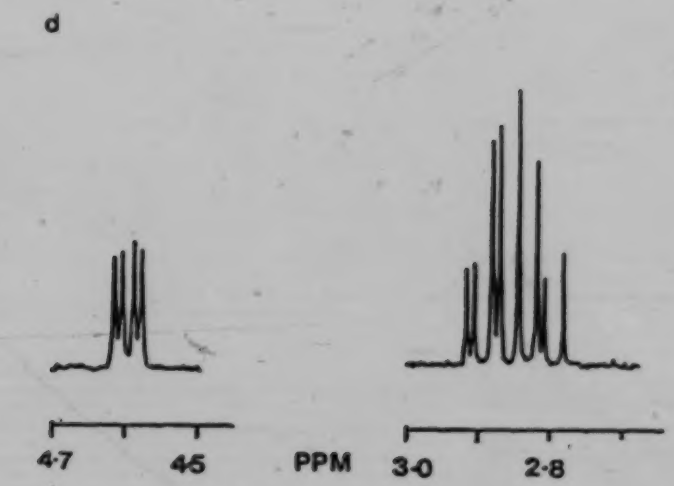


Figure 4.3. ^{1}H NMR spectra of octopamine from DBH reaction with C-2 [^{2}H]tyramine chemically derived from C-2 [^{2}H]tyrosol. *(continued)*
(d) Standard octopamine

resulting splitting pattern of the C-2 proton by these nonequivalent C-1 protons gives signals with slightly different chemical shifts. The C-2 proton is split into two sets of doublets, one at 4.64 ppm and the other at 4.62 ppm. The splitting pattern around 2.8 ppm, a multiplet, is due to the C-1 protons in the nonequivalent magnetic environment created by the chiral centre at C-2. These protons interact with each other and split each other into 2 sets of doublets, which are further split into doublets by C-2 proton, thus creating a multiplet. The signal for one of the C-1 protons is around 2.91 ppm and that of the other around 2.85 ppm. These signals of the protonated octopamine are complicated by those of C-2 deuterated octopamine, which is also present in the product. Only the C-1 protons show splitting patterns when deuterium is present at C-2 of octopamine. The splitting pattern by one of the C-1 protons with the other, a doublet, is superimposed on the signals of the protonated octopamine around 2.91 ppm. The signals of the other C-1 proton, for the deuterated octopamine species, are distinguishable and are between the signals of the protonated octopamine species centred around 2.85 ppm. These broad signals, with an upfield shift of about 0.013 ± 0.002 from the protonated octopamine, are observed for the deuterated species. No signals are seen at C-2 for deuterated octopamine since deuterium is present. This implies that the observed 4.6 ppm signal is due only to the proton at C-2 of the protonated octopamine species. About 58% protonated octopamine species was estimated from the NMR spectrum shown in Fig 4.3a, which implies that approximately 42% deuterated species was still present. As DBH is pro-*R* specific, the 42% deuterated octopamine species present must have the *S* configuration at C-2. This implies that, out of the 1.2 deuterium atoms estimated in the starting C-2 [^{2}H]tyramine, approximately 0.5 of the atoms had been incorporated at the pro-*S* position and 0.7 atoms at the pro-*R* position (Table 4.2). The resulting complex signals of the octopamine product (Fig. 4.3a) therefore indicate the nonstereospecific incorporation of solvent at C-2 of the original tyrosol in the porcine amine oxidase reaction. This is in contrast to the bovine enzyme, which has been

reported to wash-in solvent only in the pro-*R* position of the product of dopamine oxidation (9).

The solvent exchange experiment with the bovine enzyme was duplicated with tyramine. The product, tyrosol, after chemical conversion into tyramine (Fig. 4.2b) was used as the C-2 deuterated substrate for the DBH reaction. The octopamine produced was analysed by ^{1}H NMR (Fig. 4.3b). The deuterium present at the C-2 position of the tyramine (derived from bovine enzyme incubation) was cleaved off by the DBH, resulting in protonated octopamine. As DBH is pro-*R* specific when hydroxylating at C-2 of dopamine (9), deuterium could only have been in the pro-*R* position to give an octopamine product that did not contain any deuterium (Fig. 4.3b). In other words, DBH replaced deuterium in the *R* position with an -OH group, therefore labelling by bovine plasma amine oxidase at C-2 of tyramine proceeded stereospecifically into the *R* position. Estimation of the extent of labelling gave an average value of 96%, which is similar to the 95% stereospecificity reported by Farnum and Klinman (9). The octopamine in Fig. 4.3b compared very well with the standard protonated octopamine analyzed (Fig. 4.3d).

If the reaction of bovine plasma amine oxidase is pro-*R* specific at C-2 (as shown above) of tyramine, then it would be expected that a reaction involving the enzyme with C-2 dideuterated tyramine in protonated buffer would release deuterium from the *R* position, replace it with a proton, and fully retain the deuterium in the *S* position. The tyrosol produced from such a reaction is shown in Fig. 4.1c. Basically, a mixed tyrosol product, containing mostly the monodeuterated species and the diprotonated species, was produced, and this contained an estimated 1.1 atoms of deuterium. The corresponding amine from the chemical synthesis (Fig. 4.2c), was hydroxylated by DBH. The ^{1}H NMR spectrum of the octopamine product from the DBH reaction is shown in Fig. 4.3c. It is obvious from this spectrum that no signals appeared at 4.6 ppm, which is the position for C-2 proton signals, if present. The only signals, a multiplet, appeared upfield around 2.9 ppm due to the interaction between the two C-1 protons. This implied that deuterium had

been retained in C-2 at the *S* position, and that the wash-in of proton into C-2 dideuterated tyramine by bovine plasma amine oxidase had occurred only into the *R* position. Estimation of the deuterium retained was >97%, which compares favourably with the >99% retention of label observed by Summers *et al.* (7).

Stereochemistry of wash-out experiment

The wash-out experiment with porcine plasma amine oxidase and stereospecifically-labelled (2*R*)-[^{2}H]tyramine produced tyrosol, which was analyzed by ^{1}H NMR (Fig. 4.4a). The nonstereospecificity of the reaction is seen from the signals of the product. The C-1 protons (3.8 ppm) show a doublet superimposed on a triplet. The doublet, with an upfield shift of 0.02 ppm, is the major C-2 monodeuterated tyrosol, and the triplet, the minor diprotonated species. The C-2 protons show a triplet superimposed on another triplet at 2.7 ppm. The triplet due to the major monodeuterated species is shifted upfield about 0.02 ppm from the minor diprotonated species. The estimated value of deuterium retained in the alcohol product was 79%, implying about 21% protonated species was formed (Table 4.2). The nonstereospecificity of the reaction course was confirmed further in a reaction using the (2*S*)-[^{2}H]tyramine and the porcine enzyme. The alcohol product obtained from the reaction is shown in Fig. 4.4b. The predominant monodeuterated tyrosol shows a doublet for its C-1 protons, superimposed on a triplet signal belonging to the minor diprotonated tyrosol at 3.8 ppm. An upfield shift of 0.02 ppm is observed for this monodeuterated tyrosol. The two overlapping triplets near 2.7 ppm are due to the C-2 protons of both tyrosol species, and again the 0.02 ppm upfield shift due to the monodeuterated tyrosol is observed. The amount of deuterium estimated from the relative intensities of the NMR signals indicates that 62% of the original deuterium in (2*S*)-tyramine is retained and 38% is lost (Table 4.2). This establishes a nonstereospecific reaction course for tyramine at C-2. A complete nonstereospecific reaction would have resulted in an alcohol product containing an equal mixture of the monodeuterated and diprotonated

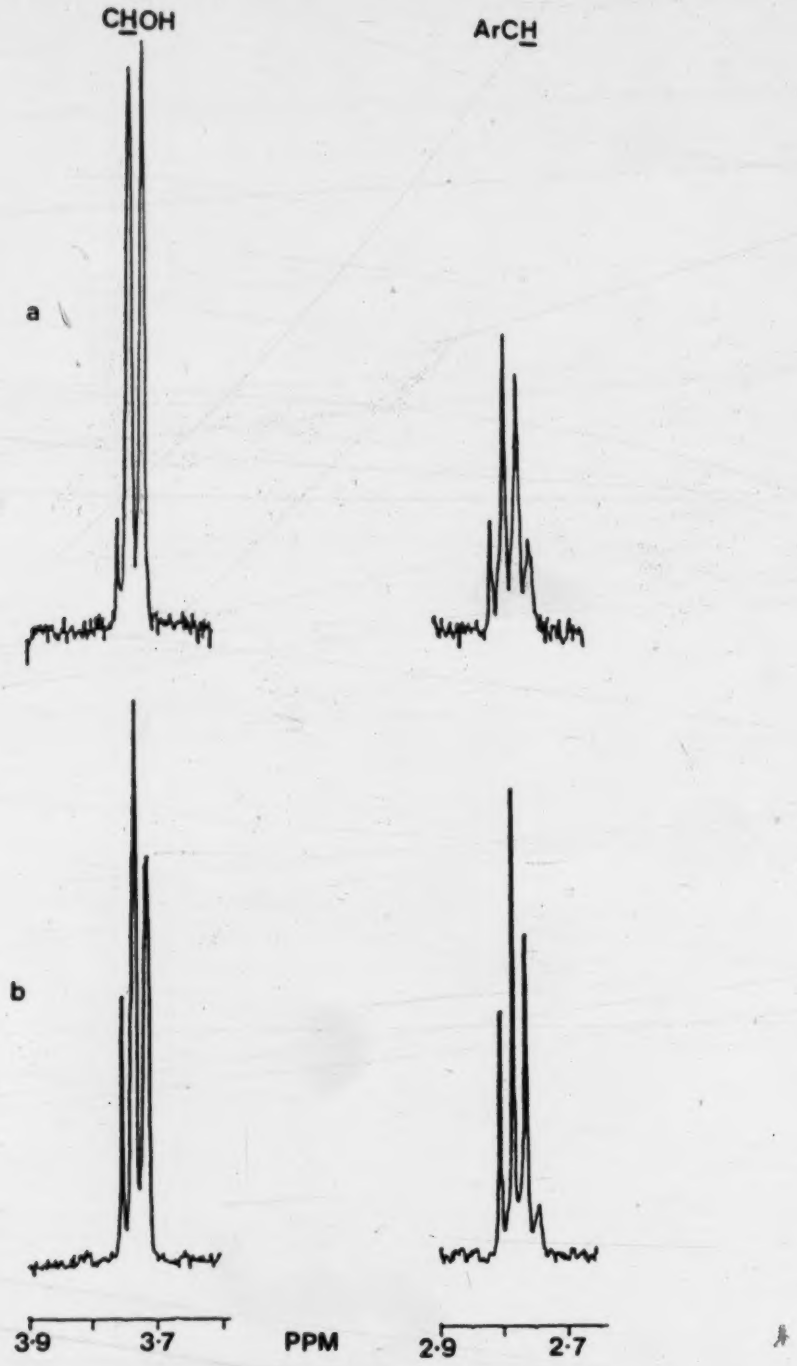


Figure 4.4. ^{1}H NMR spectra of C-2 [^{2}H]tyrosol from porcine amine oxidase incubation with C-2 [^{2}H]tyramine: wash-out.
(a) (2*R*)-[^{2}H]tyrosol
(b) (2*S*)-[^{2}H]tyrosol

tyrosols. The unequal ratios imply a partial nonstereospecificity for both the reactions proceeding via the *R* or *S* modes, resulting in products containing predominantly the deuterium species. Thus porcine enzyme exhibitis isotope effects of 3.8 for the *R* mode and 1.6 for the *S* mode of its nonstereospecific course of reaction at C-2 of tyramine (Table 4.2). The lower value for the *S* mode is an indication of a preferential abstraction of the pro-*S* proton.

In an experiment using dideuterated tyramine with the porcine enzyme in H_2O (spectrum not shown), about 0.8 proton exchanged into the C-2 position, leaving approximately 1.2 deuterium atoms present in the product alcohol. Any isotope effect obtained should reflect the lower value obtained above for the monodeuterated tyramine reaction with the porcine enzyme in H_2O. The isotope effect of 1.5 obtained in the stereochemical study with the dideuterated tyramine is comparable to the smaller value of 1.6 obtained for the *S* mode of C-2 proton exchange with the monodeuterated substrate. This suggests that proton exchange in the dideuterated substrate occurs mostly through the *S* mode, and work is in progress to elucidate the stereochemistry of the wash-in.

On the other hand the KIE on V_{max}/K_m, 1.2 (Table 4.3), obtained from the spectrophotometric measurements in the kinetic study of the C-2 diprotonated and dideuterated tyramines with the porcine enzyme, yielded a smaller isotope effect for proton exchange at C-2. This is contrary to the stereochemical results obtained above.

No real explanations can be given for the different values obtained for the kinetic and stereochemical studies, however it is important to note that the kinetic experiments involved initial rate measurements where there was negligible depletion of substrate, while the stereochemical experiments involved continuous depletion of substrate concentration.

The results obtained from both the wash-in and wash-out experiments suggest that neither of the reactions is the microscopic reverse of the other. This is evident in the number of deuterium atoms washed in at C-2 of the diprotonated tyramine (1.2 atoms) and the number washed out from the dideuterated tyramine (0.8 atom). This observation violates

Table 4.3. Kinetic parameters for the oxidation of tyramine.

	Unlabeled	*Dideutero*
K_m (mM)	1.43±0.39	1.71±0.31
V_{max} (nmole/min)	8.53±0.48	8.37±0.32
V_{max}/K_m (nmole/min·mM)	5.96±1.45	4.83±0.80
KIE on V_{max}	1.02±0.07	
KIE on V_{max}/K_m	1.23±0.36	

KIE on $V_{max} = (V_{max})^H/(V_{max})^D$

KIE on $V_{max}/K_m = (V_{max}/K_m)^H/(V_{max}/K_m)^D$

the principle of microscopic reversibility, which states that both the forward and reverse directions of a reaction should be equivalent. Farnum and Klinman (9) also reported this apparent violation of microscopic reversibility in their reaction of the bovine enzyme with C-2 labelled dopamine. They observed that enamine protonation (wash-in), which was stereospecific, was not the reverse of the imine exchange (wash-out), which was nonstereospecific (Scheme 4.2).

The nonstereospecificity of the wash-out experiment with bovine plasma amine oxidase (7,9) has been confirmed in our experiments using ^{1}H NMR for analysis (Fig. 4.5a and b). The signals for the protons are similar to those observed in Fig. 4.4a and b, for the tyrosol obtained by the porcine enzyme reaction with (2*R*)- and (2*S*)-[^{2}H]tyramines. From the relative intensities of the NMR signals, 73% deuterium was retained in the (2*R*)-[^{2}H]tyrosol with a 27% loss, while 66% was retained in the (2*S*)-[^{2}H]tyrosol with a corresponding 34% loss. This gives isotope effects of 2.7 and 1.9 for the reaction through the *R* and *S* modes, respectively (Table 4.2). A preferential abstraction of the pro-*S* proton is observed here, as was the case with the porcine enzyme. Summers *et al.* (7), however, reported an isotope effect of 1.0 for both the *R* mode (59% tritium retained, 41% lost) and the *S* mode (47% tritium retained, 53% lost) of proton abstraction. The difference here in isotope effect values may be due to the different ways of estimating the different isotope contents, one depending on relative intensities of NMR signals and the other depending on radio assay, which reflects isotope effects on V/K.

Comparison between C-1 and C-2 proton abstractions

Scheme 4.4 is a schematic representation of the stereochemical course of oxidation at C-1 and proton exchange at C-2 of tyramine involving the cofactor topa. In this scheme, after the substrate nitrogen binds the carbonyl group in the cofactor topa, the pro-*R* hydrogen is abstracted by the basic residue at the active site of the bovine enzyme, to form the imine. The next step is always the abstraction of the pro-*S* hydrogen at C-2 of tyramine.

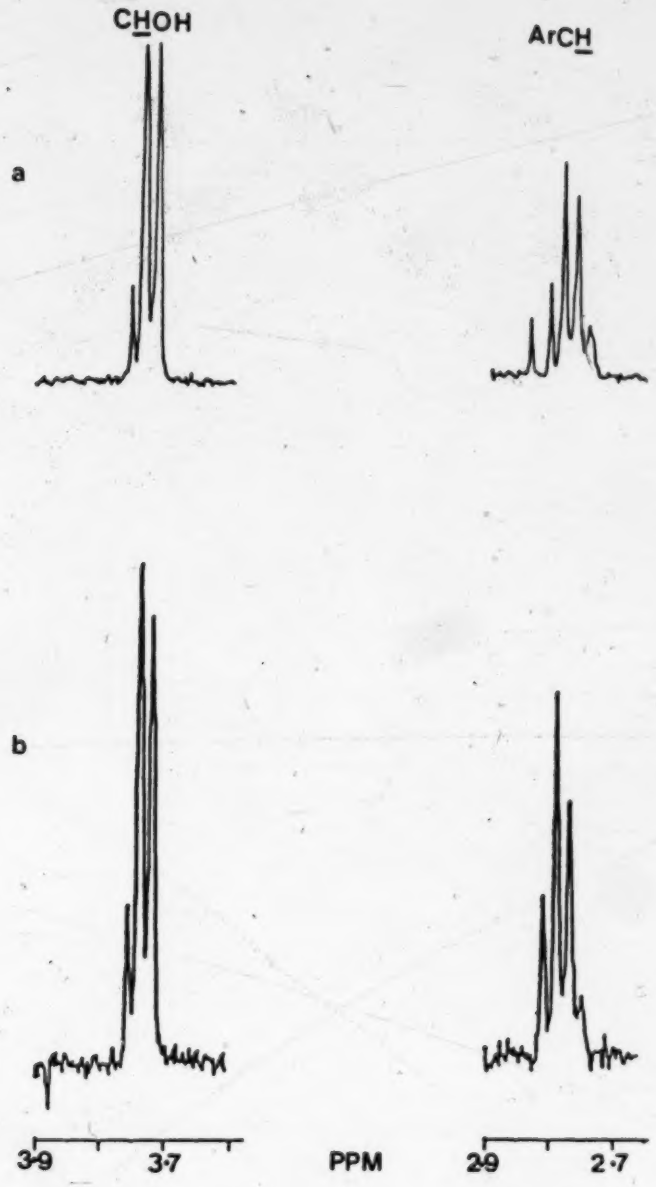
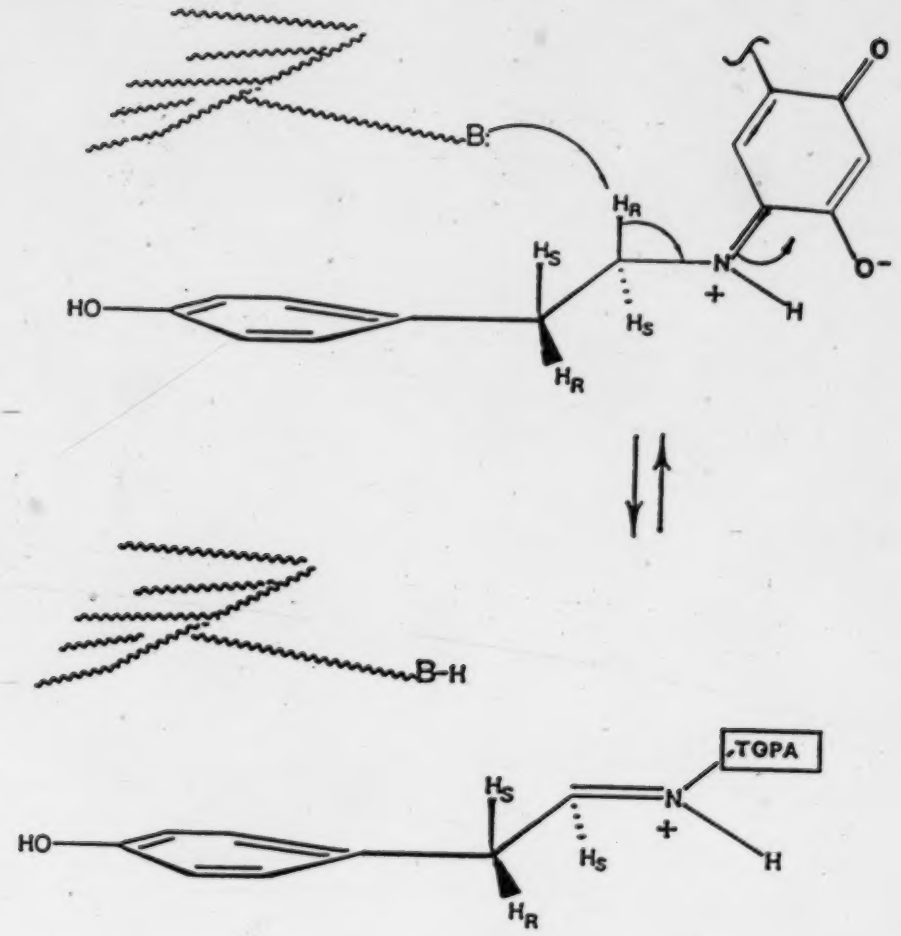
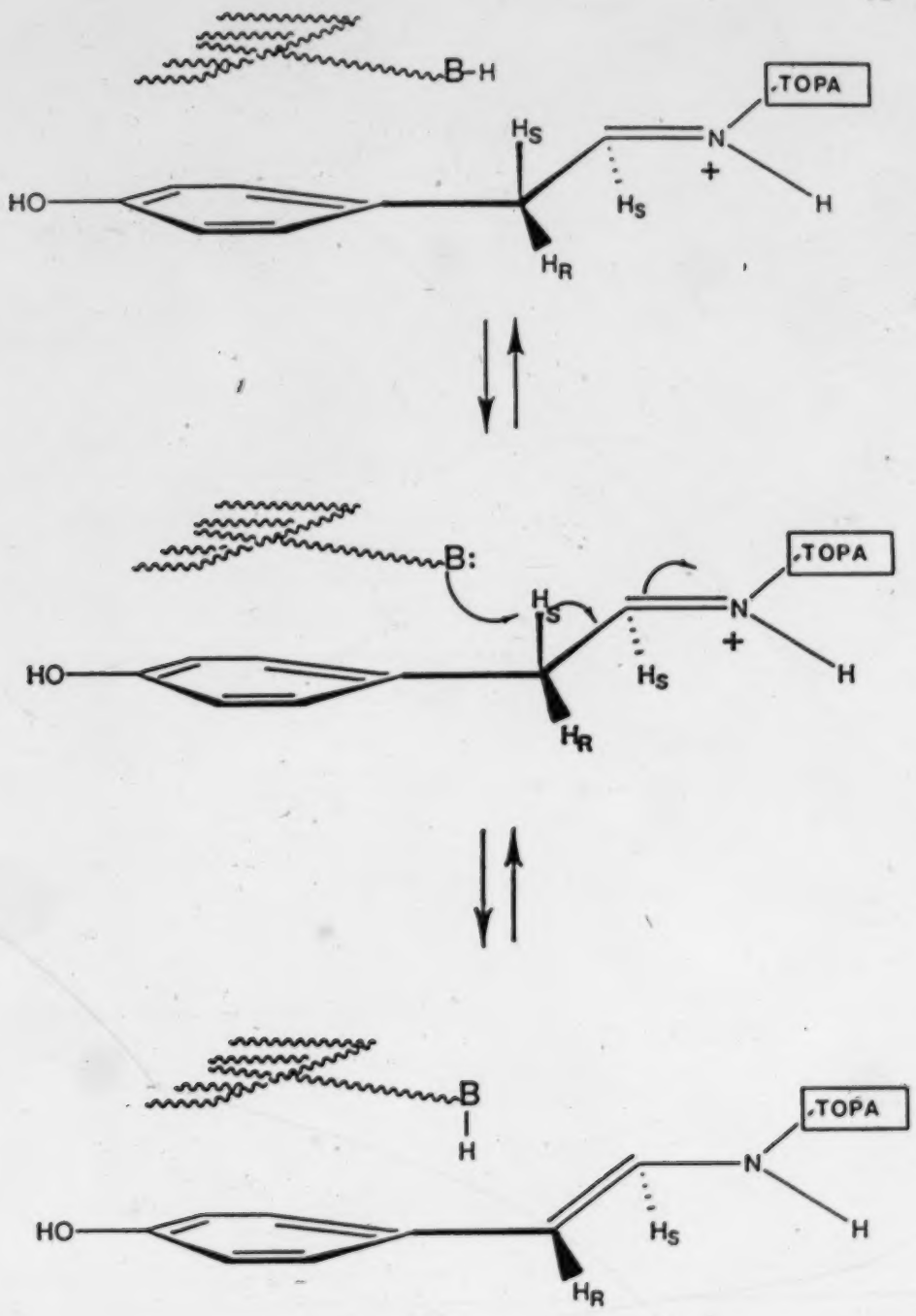


Figure 4.5. ^{1}H NMR spectra of C-2 [^{2}H]tyrosol from bovine amine oxidase incubation with C-2 [^{2}H]tyramine: wash-out.
(a) (2*R*)-[^{2}H]tyrosol
(b) (2*S*)-[^{2}H]tyrosol



Scheme 4.4. Schematic representation of proton abstraction at C-1 and C-2 of tyramine.



Scheme 4.4. Schematic representation of proton abstraction at C-1 and C-2 of tyramine. *(continued)*

This is possible as the basic residue lies above the plane of the substrate. As reported by Farnum and Klinman (9) and as observed in studies by Coleman *et al.* (6), dopamine and tyramine have two binding modes in the active site of the bovine enzyme, such that the pro-*S* hydrogen can also be abstracted at C-1 of the substrate. This means that, after substrate binding, the substrate orients itself (bond rotation) so that the pro-*S* hydrogen can be abstracted. As there is one basic residue involved in abstracting this pro-*S* hydrogen at C-1, the substrate is oriented in such a way as to result in the pro-*R* hydrogen being abstracted at C-2. The same argument applies to tyramine.

The evidence for this single basic residue at the active site of the bovine enzyme was from kinetic and pH dependent studies at both C-1 and C-2 positions of dopamine (16), where a similar pK_a value was measured. Hence, in the oxidation of dopamine by bovine plasma amine oxidase, as reported by Farnum and Klinman (9), a single base catalyzes the C-H bond cleavages at C-1 and C-2 in a syn manner. The report also indicated that a mirror-image catalysis took place in which dopamine had two functional binding orientations on the enzyme surface, leading to the nonstereospecificity of the reaction a C-1 and also the nonstereospecificity of proton removal at C-2. In comparison, the pro-*R* specificity at C-1 of tyramine and dopamine with porcine plasma amine oxidase (6) suggests that only a single catalytically-active binding orientation exists on the enzyme surface for the substrates.

The nonstereospecificity of the incorporation of label at C-2 of substrate by the porcine enzyme may implicate either a syn or an anti manner of C-H bond cleavage at C-1 and C-2. Although more experiments are required to prove the relationship between the C-H bond cleavage at C-1 and C-2 of tyramine, one can speculate that a syn relationship would mean the presence of one base removing the protons, whereas an anti relationship of proton removal would mean the presence of two bases on the enzyme molecule.

As previously reported by Coleman *et al.* (6), the porcine amine oxidase is pro-*R* specific at C-1 of both dopamine and tyramine. In an analogy to the arguments above for

the bovine enzyme, using Scheme 4.4, binding of tyramine to the cofactor in the active site of the porcine enzyme occurs in such a way as to allow only the pro-*R* abstraction of the C-1 proton. Following this initial abstraction, only the pro-*S* proton at C-2 should be abstracted for a similar syn manner in the bond cleavages at C-1 and C-2. However, the stereochemical results at C-2 indicate a nonstereospecific pro-*R* and pro-*S* abstraction of protons, which means that, in addition to the pro-*S* proton, the pro-*R* proton at C-2 is also abstracted. For this stereochemical course to occur, in a syn manner of cleavage, i.e. cleavage of C-1 (pro-*R*) and C-2 (pro-*R*) protons, a rotation about the C-1 and C-2 bond most likely must take place after imine formation (i.e. after the C-1 pro-*R* proton is abstracted) to enable enamine formation. On the other hand, if an anti relationship of proton removal existed, then two bases at the active site of the enzyme are possible, such that one basic residue lying above the plane of the substrate in the active site of the enzyme will abstract the C-1 (pro-*R*) proton and the other one, lying below the plane, will abstract the C-2 (pro-*R*) proton. This would also mean that the C-2 pro-*S* proton will be abstracted by the base lying below the plane only after a bond rotation at C-1 and C-2.

Studies in 1976 by Lindstrom *et al.* (14) and Olsson *et al.* (15) suggested the involvement of a single basic residue in the reaction of porcine enzyme and substrate. Farnum *et al.* (16), in an analogy to the bovine enzyme's reaction, suggested that an ionized form of a catalytic residue in the active site of the porcine enzyme was responsible for the enzymatic catalysis. These observations may implicate one basic residue at the active site of porcine plasma amine oxidase. This would suggest that the C-H bond cleavage at C-1 and C-2 of tyramine could be occurring in a syn fashion. Stronger evidence to support or refute this suggestion may come from molecular modelling experiments and from kinetic studies involving the oxidation of substrates labelled at C-1 and pH dependent studies of the cleavage of proton from C-2 labelled substrates.

The stereospecificity of wash-in and nonstereospecificity of the wash-out reactions of the bovine enzyme are in contrast to the nonstereospecificity of both reactions for the

porcine enzyme. The wash-out reaction proceeds before wash-in, such that in the case of the bovine reaction, after the proton is abstracted at C-2, reprotonation always occurs at one face of the double bond in the enamine, which leads to the stereospecific wash-in. This stereospecific reprotonation or wash-in, reported by Farnum and Klinman (9), is the consequence of a rotation about the C-N bond formed after the imine tautomerizes to the enamine (Scheme 4.4). The observations that the enamine protonation was not the microscopic reverse of the imine exchange (Scheme 4.2) appeared to violate the principle of microscopic reversibility (9), which states that the forward and reverse directions of a reactions should be equivalent.

In contrast to the bovine enzyme, reprotonation in the porcine enzyme reaction occurs on either face of the double bond of the enamine, which leads to the nonstereospecific wash-in of the reaction. This difference in reprotonation may be controlled by the binding and orientation of the C-1 protons at the active site of the enzymes. The stereochemical studies at C-2 (wash-out) of tyramine using the porcine enzyme gave isotope effect values of 3.8 (2*R* mode) and 1.6 (2*S* mode), with the preferential processing of the substrate through the *S* mode. This contrasts to the preferred pro-*R* wash-in by the enzyme (i.e. 0.7 label into *R* position and 0.5 into *S* position). As was observed in the bovine enzyme reaction (Scheme 4.2), the reversibility of the enamine protonation and imine exchange is nonequivalent, hence the apparent violation of the principle of microscopic reversibility.

Thus the 1*R* and 1*S* modes of proton abstraction at C-1 of tyramine by the single base in bovine plasma (9) always lead to a 2*S* and 2*R* abstraction of proton, respectively, at C-2 and a stereospecific pro-*R* reprotonation of the enamine formed. On the other hand, the pro-*R* specific abstraction of proton at C-1 of tyramine by the single basic residue (14-16) in porcine enzyme may orient the substrate in such a way that either of the C-2 protons (preferably the 2*S*) is abstracted nonstereospecifically, leading to reprotonation which also occurs nonstereospecifically.

Studies undertaken independently by researchers (17-20) have proposed that the cofactor in the bovine plasma amine oxidase was pyrroloquinoline quinone (PQQ). Although pyridoxal phosphate was discounted long ago as the cofactor, some recent publications still adhere to it (21-23). Another recent publication by Klinman *et al.* on the bovine enzyme has implicated the compound 6-hydroxydopa (topa) as the active site cofactor (4). It was also thought that the porcine enzyme contained PQQ, yet another report has argued against this (23). Moreover, preliminary and unpublished reports from Klinman's laboratory indicate that the active site cofactor of the porcine enzyme is similar to that found in the bovine enzyme. This then raises the question of why two different stereochemistries of oxidation of the same substrate by the different sources of enzymes occur. An explanation may lie in the orientation of residues at the active site relative to the orientation of the bound substrate, as mentioned earlier. The proposal of topa as the active site carbonyl species would still form the initial Schiff base with substrate, similar to the proposed aminotransferase mechanism for copper oxidases (3,9) shown in Scheme 4.4. After the formation of the Schiff base between the cofactor carbonyl and substrate amine, the base-catalyzed proton abstraction from C-1 follows, resulting in an imine species. This allows the cofactor to function as an electron sink during the catalysis, which is analogous to the mechanism of pyridoxal phosphate.

It is clear from these studies that the enamine pathway, off the main catalytic route, has been conserved in the enzymes, however in the competition for hydrolysis versus the exchange rate, a variation occurs which may be due to some intrinsic factor of the enzymes, resulting in the different extents of deuterium incorporated at C-2.

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5. Concluding Remarks

The stereochemical studies carried out in this research have resulted in interesting observations about copper amine oxidases (E.C. 1.4.3.6) in general.

It has been shown that this class of enzymes does not possess a uniform stereochemical course in its reaction pathway for the abstraction of the C-1 proton of substrate during oxidative deamination. Bovine plasma amine oxidase is pro-*S* specific for benzylamine, *p*-hydroxybenzylamine and 3-methylbutylamine, but nonstereospecific for dopamine and tyramine when abstracting the C-1 protons of these substrates. This observed nonstereospecificity of the bovine enzyme reaction with the different substrates is attributed to two different binding modes at the active site of the enzyme. The larger isotope effect values of 15.6 and 6.7 for the *S* modes of dopamine and tyramine oxidations suggest that the cleavage of proton by the bovine enzyme proceeds with a preference for the *R* mode of proton abstraction at C-1 of the same substrates, such that reduced isotope effect values of 5.6 and 4.5 are observed for dopamine and tyramine. Likewise, the rabbit enzyme is nonstereospecific during the abstraction of the C-1 protons of tyramine. An isotope effect value of 4.6 was obtained for the *S* mode and 2.7 for the *R* mode, which also indicates that the rabbit enzyme has a preference for the *R* mode of tyramine oxidation. On the other hand, the porcine enzyme is pro-*R* specific in its reaction when abstracting the C-1 proton of dopamine and tyramine. In contrast to these stereochemistries of bovine, rabbit and porcine enzymes involving the substrates dopamine and/or tyramine, the pea seedling amine oxidase is pro-*S* specific during the cleavage of the C-1 proton of dopamine and tyramine. These different stereochemistries at C-1 of the substrates are the first known example where a group of enzymes in the same class possesses different stereochemical courses in exactly the same reaction with the same substrates.

Another observation from this study was the incorporation of label (^2H_2O solvent) into the C-2 position of dopamine and tyramine by the enzymes used in this research. All the plasma enzymes, namely bovine, rabbit and porcine, incorporated ^2H (deuterium) into

the C-2 position of the substrates. Incorporation occurred to different extents for these enzymes, which meant that the rate of enamine formation and hydrolysis during the reaction was proceeding at different extents for the enzymes. The inability of the pea seedling enzyme to incorporate label into the C-2 position meant that this solvent exchange pathway was off the main enzyme pathway for oxidative deamination. As such, hydrolysis of the imine intermediate, common to all the enzymes, proceeded much faster for the pea seedling enzyme such that it bypassed the imine-enamine tautomerization pathway. This tautomerization pathway lived long enough to cause exchange of solvent into the C-2 position for the plasma enzymes.

For the bovine plasma amine oxidase, the pro-*R* specific incorporation of label into C-2 of dopamine was also found to occur with tyramine. This was contrary to the non-stereospecific wash-in of label into tyramine by the porcine plasma amine oxidase, an observation which seems to reiterate the point that these two enzymes, although they are in the same class, behave differently. The only similarity has been the nonstereospecific abstraction of the C-2 protons from tyramine by both enzymes. The slightly higher kinetic isotope effect value for porcine enzyme (3.8 for *R* mode; 1.6 for *S* mode) as against that for bovine enzyme (approximately 1 for *R* and *S* modes) is an indication that the imine-enamine tautomerization common to either enzyme stays long enough in the porcine enzyme reaction pathway to bring about solvent exchange into both C-2 positions.

As bovine enzyme has shown pro-*S* specificity for an aromatic amine with one methylene (-CH_2) group (benzylamine) and for an aliphatic amine (3-methylbutylamine), some future research considerations would likely include:

i. Studies on the stereochemistry of porcine plasma amine oxidase with these substrates.

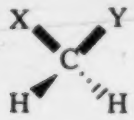
ii. Investigations to support the presence of a single base at the active site of the porcine enzyme by studying (a) kinetics of oxidation at C-1 of tyramine and (b) the pH dependence of exchange at C-2 of tyramine.

iii. Studies screening plasma amine oxidases for an enzyme with pro-*S* specificity at C-1 for tyramine, and for other plasma sources with pro-*R* specificity.

iv. The exploration of evolutionary relationships between the enzymes by comparing their amino acid sequences.

Appendix 1

(a) Prochiral centre:



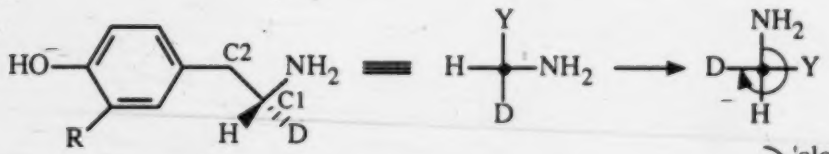
i. C: prochiral carbon centre has two identical hydrogen atoms and two other different atoms attached.
ii. pro-*R* (or pro-*S*) hydrogen: one of the chemically identical hydrogens at the prochiral centre.

(b) Chiral centre:

i. C: chiral carbon centre has four different atoms attached (Y>X>D>H).
ii. pro-*S*: deuterium substitution that generates a chiral centre with an *S* configuration (I).
iii. pro-*R*: deuterium substitution that generates a chiral centre with an *R* configuration (II).

(c) Chiral amines:

i.

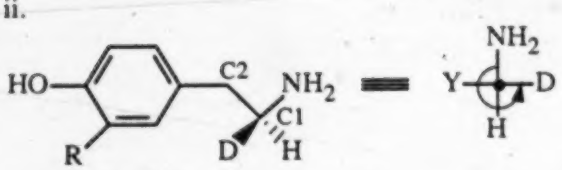


'clockwise direction'

(1*R*)-[^{2}H]

i.e. D in *R* position
(Cahn-Ingold-Prelog system)
see reference*

ii.

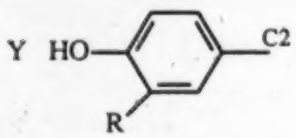


'anticlockwise direction'

(1*S*)-[^{2}H]

i.e. D in *S* position
(Cahn-Ingold-Prelog system)
see reference*

• C1 chiral centre



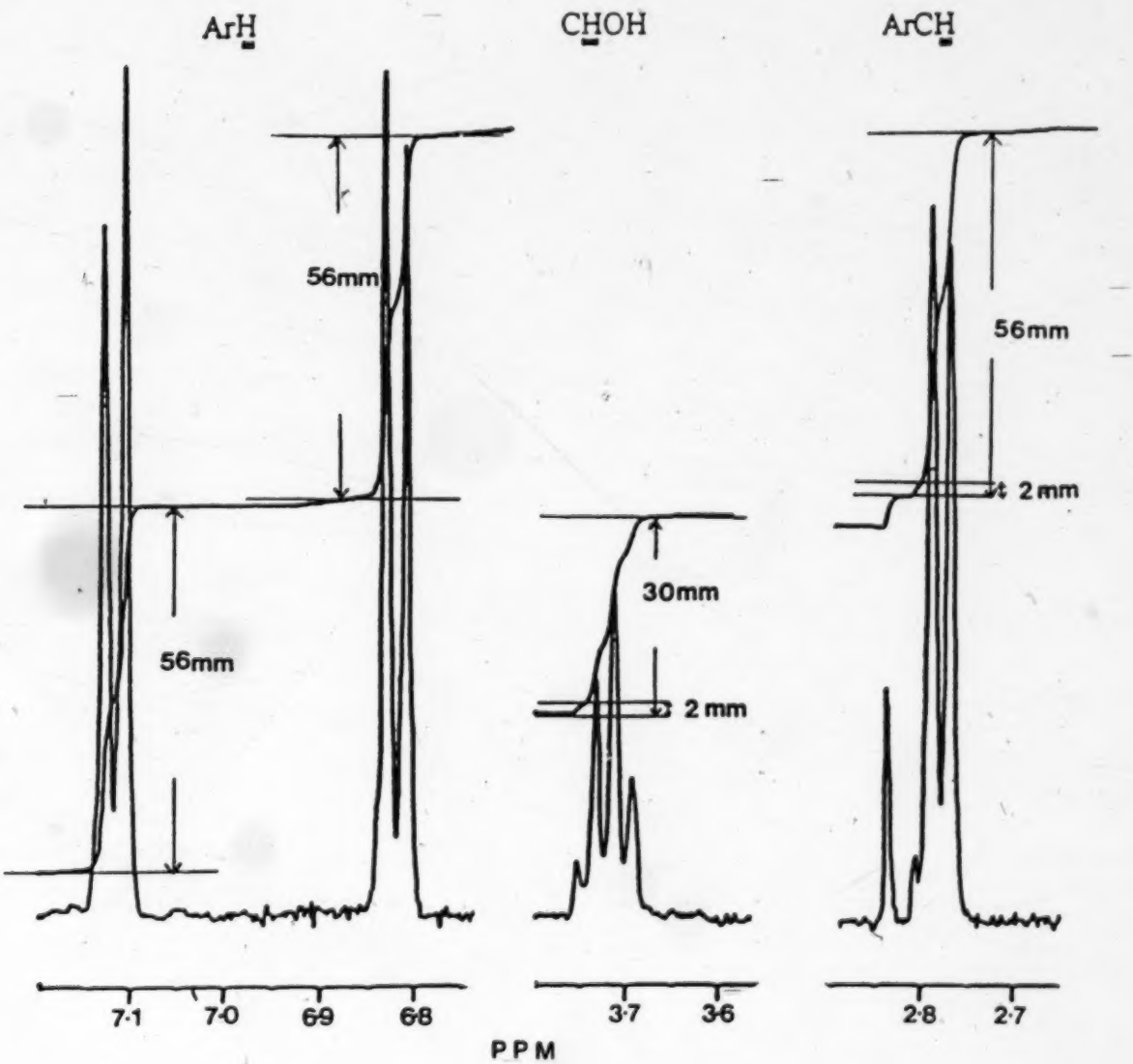
R = H : [^{2}H]tyramine

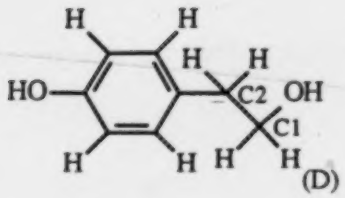
R = OH : [^{2}H]dopamine

* For stereochemical designations refer to:
Stereochemistry of enzymatic reactions (1985). In: 'Enzyme Structure and Mechanism'. Fersht, A. (ed.), 2nd edition. W.H. Freeman and Company, New York.

Appendix 2

^{1}H NMR spectrum of tyrosol from a nonstereospecific enzyme reaction: estimation of deuterium content.





(D)

		Group	
	ArH	CHOH (C1)	ArCH (C2)
Number of protons for signal	4	1	2
Height of wing, A (mm) (start of triplet for diprotonated tyrosol)	- -	2	2
Total height, B, of signal (mm)	112	30	56
Height of single proton, C (mm) (calculated from ArH or ArCH)	28		28

Absolute value of triplet in 1:2:1 ratio (D) = 4

Number of different species present at CHOH (E) = 2

Estimating % diprotonation for mixed alcohol product:

[1] From ArCH (C2): $\frac{A \times D}{B} \times 100$

$$\frac{2 \times 4}{56} \times 100 = 14.3\%$$

[2] From CHOH (C1): $\frac{A \times D}{E \times B} \times 100$

$$\frac{2 \times 4}{2 \times 30} \times 100 = 13.3\%$$

Average of % diprotonation (diprotonated alcohol) = $13.8 \pm 0.7\% \approx 14\%$

$\therefore$ % deuteration remaining in product (monodeuterated alcohol) $\approx 86\%$